

**PESTICIDES IN AQUATIC SYSTEMS: AN ECOLOGICAL AND EVOLUTIONARY  
PERSPECTIVE**

by

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# **PESTICIDES IN AQUATIC SYSTEMS: AN ECOLOGICAL AND EVOLUTIONARY PERSPECTIVE**

Jessica Hua, PhD

University of Pittsburgh, 2014

Disturbances play important roles in shaping ecological and evolutionary processes. By using disturbances to perturb natural systems, biologist can both develop generalizable predictions about how disturbances alter natural systems as well as utilize disturbances as a tool to test ecological and evolutionary theory. Using pesticide disturbances in aquatic systems, the first half of this thesis integrates ecology, evolution, and toxicology to develop predictions about the consequences of pesticides in aquatic communities and patterns of pesticide tolerance across populations of wood frogs (*Lithobates sylvaticus*). Towards this goal, I first conducted a mesocosm study tracking the direct and indirect effects of four insecticides—applied individually and as a mixture— across 18 weeks and demonstrated that insecticides applied individually and in a mixture have complex direct and indirect consequences on aquatic system response and recovery not predicted by traditional laboratory tests. Second, I investigated the potential for cross-tolerance in non-target populations of wood frogs and demonstrate that amphibian populations with tolerance to one pesticide may be cross-tolerant to many other pesticides.

The second half of this dissertation uses pesticides as a tool, to test theoretical predictions about the role of phenotypic plasticity in evolutionary innovation to novel environments. I investigated whether natural populations of wood frogs can respond plastically to pesticides (i.e. by inducing increased tolerance) and whether there is evidence supporting the process of genetic assimilation. This study is the first to demonstrate that sublethal and ecologically relevant concentrations of a

common insecticide can, within the same generation, induce adaptive tolerance in amphibians and the population-level patterns of inducibility are consistent with predictions of genetic assimilation. Induced pesticide tolerance would be particularly beneficial to non-target species if it were to confer increased tolerance not only against the pesticide it first experienced, but also against many other pesticides (e.g., induced cross-tolerance). Using wood frogs, the final chapter of this dissertation demonstrated the phenomenon of induced cross-tolerance and suggests that cross-tolerance is not limited to insecticides that share mode of action. Overall, the inducible tolerance and cross-tolerance findings suggest that phenotypic plasticity may play a role in shaping patterns of species abundance in nature.

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## **PREFACE**

I would like to start by thanking the scientists that played such a significant role in the development of this thesis as well as my development as a researcher. First and foremost, I must thank my advisor, Dr. Rick Relyea. Without a doubt, I would not be here if it were not for him. He took a chance on me, an unsuspecting pre-med student who never wanted to leave Texas, and through his relentless effort, patience, and guidance, transformed me into not only a broad thinking scientist but truly into a better person. His intense commitment to putting his students first will always be an inspiration to me and will be the model that I am committed to maintaining in my future pursuits. Early in my graduate career, Rick told me “success favors the prepared mind.” Throughout my years at Pitt, I worked really hard attempting to maintain this “prepared state” but what did I not always notice was that Rick was constantly working hard behind the scenes to create an environment that would allow his students to grow and to be successful. He worked to build widespread collaborations that allowed me the opportunity to collaborate with amazing researchers throughout the U.S. as well as abroad. He fostered a team environment in the lab which allowed us to carry out large-scale experiments not possible elsewhere. These are just a few of the incredible unique opportunities that Rick worked so hard to make available to his students. Thus, my future success will always be directly linked to his efforts.

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Finally, words could not describe how thankful I am for my family. Everything I am and have is because of their efforts. From my father, I learned how to work hard and to never compromise on pursuing my potential. Whether it was through extra tutoring, piano, violin or figure skating lessons, throughout my life, my mother put it upon herself to do all that she could to provide me with every opportunity to be successful. My parents escaped from Vietnam after the war and landed on American soil with a single suitcase. From this starting point, they rebuilt their lives from scratch with the single goal of allowing my sisters and I the opportunity that had eluded him. Having gone through the hardships of living through a war, my parents have

developed a unique philosophical viewpoint that without a doubt shapes the way I see the world as well as how I pursue my work as a scientist. Thus, my achievements are as much mine as they are yours. I love you both!

## **1.0 INTRODUCTION**

Disturbances (i.e. discrete events in time that disrupt ecosystems, communities or population structure and change resource or substrate availability of the physical environment; Picket and White 1985) play significant roles in shaping ecological and evolutionary processes in nature. By using disturbances to perturb natural systems, biologist can both develop generalizable predictions about how disturbances alter natural systems as well as utilize disturbances as a tool to test ecological and evolutionary theory. Accordingly, this thesis first integrates ecology and evolution to develop generalizable predictions about the complex consequences of disturbance in complex communities and across populations (Chapters 2 and 3). Second, using disturbances as a tool, this thesis also tests theoretical predictions about the role of phenotypic plasticity in evolutionary innovation to novel environments (Chapters 4 and 5).

To address these aims, this thesis focuses on pesticide disturbances in aquatic systems as a model. Pesticides are of particular relevance as their use is projected to increase drastically in the next few decades affecting a wide diversity of natural systems (Laurance 2001). Aquatic systems are of specific concern as pesticides can enter into these systems via direct application as well as drift or run-off (Grube et al. 2011). Finally, pesticides are an effective model disturbance due to their diverse toxicity, mode of action, and chemical classification (Clements and Rohr 2009).

In Chapter 2, I begin by integrating ecology and toxicology to develop predictions about the effect of pesticides in complex aquatic communities. Toxicologists have traditionally relied on short-term, single-species laboratory tests to assess pesticide toxicity (Hammond et al. 2012). While this approach identifies the direct toxicity of insecticide on individual species, it overlooks the ecological complexity of natural systems potentially leading to limited or misleading predictions about the effects of pesticides (Fleeger et al. 2003). To address this limitation, I conducted a mesocosm study tracking the direct and indirect effects of four insecticides—applied individually and as a mixture—across 18 weeks. This work is in collaboration with Dr. Rick Relyea and has been accepted for publication in *Environmental Pollution*.

In addition to the ecological consequences of pesticides, evolutionary responses to pesticides are also overlooked in traditional studies of toxicology. The evolution of pesticide resistance in target species has become a severe problem costing over \$1.5 billion each year (Georghiou 1990). Further, cross-tolerance, tolerance to multiple pesticides, is frequently observed in pest species (Alou et al. 2010). Despite the evidence for cross-tolerance in pest species, cross-tolerance and its implications for non-target organisms have been largely overlooked. Chapter 3 explores the potential for cross-tolerance in non-target populations of wood frogs. This work was conducted in collaboration with Dr. Aaron Stoler, Dr. Rickey Cothran, and Dr. Rick Relyea and is published in *Environmental Toxicology and Chemistry* (Hua et al. 2013).

Next, considerable effort has sought to understand the mechanisms behind the evolution of pesticide resistance, but this understanding has almost exclusively focused on targeted pest species (Jansen et al. 2011b) and is strongly biased towards the Darwinian perspective (i.e. genetic changes in a population due to natural selection; Lopes et al. 2008). Despite increasing



interest in the role of phenotypic plasticity in shaping evolutionary processes, the role of plasticity in the evolution of pesticide resistance has been almost completely ignored. Theory predicts that by revealing hidden genetic variation, phenotypic plasticity can allow organisms to persist thereby setting the stage for evolutionary processes to occur (Schlichting and Wund 2014). Further, according to theory, induced adaptive phenotypes can over time become fixed through the process of genetic assimilation (Crispo 2007). While theoretical research and laboratory selection experiments support the potential role of plasticity in evolutionary innovation, there are few attempts at determining whether these patterns occur in nature (Crispo et al. 2010). Chapter 4 investigates whether natural populations of wood frogs can plastically respond to pesticides (i.e. by inducing increased tolerance) and whether there is evidence supporting the process of genetic assimilation. This work was conducted in collaboration with Dr. Nathan Morehouse and Dr. Rick Relyea and is published in *Evolutionary Applications* (Hua et al. 2013)

Finally, given the enormity of available pesticides (USEPA 2007) induced pesticide tolerance would be particularly beneficial to non-target species if it were to confer increased tolerance not only against the pesticide it first experienced, but also against many other pesticides (e.g., induced cross-tolerance). Chapter 5 investigates whether wood frogs can induce tolerance to not only single pesticides but can induce cross-tolerance to multiple pesticides. This work was conducted in collaboration with Devin Jones and Dr. Rick Relyea and is accepted for publication in *Environmental Science and Technology*.

To sum, as human populations continue to grow, understanding and making predictions about the contribution of anthropogenic disturbances to biological processes will continue to be an important challenge for biologists. In addition to the applied implications, studying

anthropogenic disturbances also allow biologists unique and diverse tool by which to contribute to the broader fields of ecology and evolution.

## **2.0 CHEMICAL COCKTAILS IN AQUATIC SYSTEMS: PESTICIDE EFFECTS ON THE RESPONSE AND RECOVERY OF >20 ANIMAL TAXA**

### **2.1 INTRODUCTION**

Natural systems are exposed to a number of disturbances that shape species abundance and diversity. In particular, insecticides represent a common anthropogenic disturbance to ecological systems (Grube et al. 2011). The consequences of insecticides are wide reaching, spanning all biological organization levels and broad temporal scales (Picket and White 1985). To understand the relative contribution of insecticides in shaping natural systems, we not only need to identify generalities across different insecticides (both within and across insecticide classes) but also across time and ecological levels. Further complicating this issue is that natural systems are often exposed to multiple insecticides that can lead to unanticipated additive, antagonistic, or synergistic interactions (Chèvre et al. 2005; Daly et al. 2007; Smalling et al. 2012). As insecticide use continues to increase, developing generalizations about how natural systems respond to individual insecticides or combinations of multiple insecticides is an important contemporary challenge (Pimentel 2005; Turner 2010; Puccinelli 2012).

Given the large number of different insecticides and their mixtures in natural systems, toxicologists have traditionally relied on short-term, single-species laboratory tests to determine the lethal concentration of an insecticide that causes 50% of a population to die (i.e. LC50

values; Faust, 2000; Hammond et al., 2012; Jones et al., 2009). While this reductionist approach has been helpful in understanding the direct consequences of insecticide on individual species, a growing number of studies (chronic tests; community mesocosm studies) have demonstrated that insecticides can also have indirect cascading effects at the population and community levels (Fleeger et al. 2003; Relyea and Hoverman 2006; Peters et al. 2013). Focusing solely on single-species, direct toxicity values over short time periods (1 to 4 d) can lead to limited or misleading conclusions about the effects of insecticides. Thus, we need studies that track the direct and indirect consequences of insecticides across multiple levels of biological organizations to develop generalizations about how these chemicals can alter aquatic systems (Kefford et al. 2005).

Despite the growing number of studies examining the short-term direct and indirect consequences of insecticides, our understanding still lacks much of the complexity of natural systems. Since a large number of chemicals are applied, we need to determine the generalizability of the direct and indirect consequences of different insecticides at different levels of ecological complexity. Moreover, most insecticides are designed to act immediately and degrade quickly (Newman 1992), but these short-term consequences can potentially lead to unanticipated lethal or sublethal effects on communities that may last long after the insecticide has degraded (i.e. lag effects); to address the long-term effects of insecticides, we need to temporally extend our monitoring efforts. Finally, aquatic systems are commonly exposed to complex mixtures of insecticides that can interact in unpredictable, non-additive ways (Belden et al. 2007). Determining the prevalence of these non-additive interactions has significant conservation and ecological implications.

To address these issues, we created complex aquatic mesocosms containing over 20 animal taxa. Using one-time insecticide applications at low concentrations, we exposed these mesocosms to four common insecticides applied as a mixture and applied individually at additive and substitutive concentrations. We then tracked the direct and indirect population and community responses for 18 wks by measuring the response and recovery of animal taxa and the associated changes in several abiotic variables.

## **2.2 METHODS**

### **2.2.1 Pesticide background**

We chose to work with four commonly applied insecticides: chlorpyrifos, diazinon, endosulfan, and malathion (Aston and Seiber 1997; Gilliom 2007; Grube et al. 2011). Chlorpyrifos, diazinon, and malathion belong to the same chemical class (organophosphates) and have similar modes of action (acetylcholine esterase inhibitor; Brown, 2005). In contrast, endosulfan belongs to the organochlorine class and is a GABA inhibitor (Table A1). We chose these insecticides with the intention to make comparisons between insecticides that share and differ in their chemical properties. All four insecticides are used in agricultural, residential, and public pest-control and they occur in water bodies via direct application and via indirect accidental run-off (Gilliom 2007).

### 2.2.2 Experimental design

To investigate the effects of separate and combined insecticides on aquatic systems, we carried out an 18-wk mesocosm study at the University of Pittsburgh's Pymatuning Laboratory of Ecology. We used a completely randomized design that contained 11 treatments: a negative control (water), a solvent control (ethanol), four insecticides (chlorpyrifos, diazinon, endosulfan, and malathion) applied separately at a nominal concentration of 10 ug/L (additive concentration), four insecticides applied separately at a nominal concentration of 40 ug/L (substitutive concentration), and a mixture treatment that combined 10 ug/L of all four insecticides for a total concentration of 40 ug/L. We replicated the 11 treatments four times for a total of 44 experimental units. For additional details on methods, see Supplementary Information (S.I.) 1.0.

The experimental units were plastic, 1200-L cattle watering tanks filled with ~825 L of well water on 8 to 12 April 2009. All tanks were covered using 60% shade cloth to prevent organisms from entering or leaving. This level of shade still allows high levels of primary productivity. Three days after the tanks were filled (15 April), we added 25 g of rabbit chow and 300 g of dry leaves (primarily *Quercus* spp.) to provide nutrients and additional substrate for periphyton. The following day (16 April), we collected pond water from three nearby ponds and added equal aliquots to each mesocosm to provide a natural source of algae and bacteria. We placed four ceramic tiles (15 x 15 cm) on the north side of each mesocosm to serve as periphyton samplers. On 20 April and 1 May, we collected zooplankton from four local ponds using a 30-micron zooplankton tow and added equal aliquots of the zooplankton/ pond water mix to each mesocosm (For additional details see S.I. 1.1). After adding the algae and zooplankton, we let the mesocosms sit for an additional 22 d to allow the algae and zooplankton to grow.

We then added two species of detritivores and three species of snails to the mesocosms. For the detritivores, we collected amphipods (*Crangonyx pseudocracilis*) and isopods (*Asellus aquaticus*) from two nearby wetlands and added 20 similar-sized individuals of each species to every mesocosm on 22 and 23 May. For snails we added 5 egg masses of each species to the mesocosms (for additional details see S.I. 1.2).

To mimic natural amphibian assemblages and densities (Werner et. al 2009), we added six species of amphibians to each mesocosm over time. We collected at least 11 newly oviposited egg masses for each species (Table A2). Egg masses were hatched in 200-L wading pools and fed rabbit chow ad libitum after hatching. We added 15 tadpoles of each species to the mesocosms. We began by adding four species of spring-breeding tadpoles 43 days after the tanks were filled (24 May): wood frogs (*Lithobates sylvaticus* [*Rana sylvatica*]), leopard frogs (*L. [R.] pipiens*), American toads (*Anaxyrus* [*Bufo*] *americanus*), and spring peepers (*Pseudacris crucifer*). All spring-breeding amphibians were selected from a mixture of all clutches, which were all early in development (Table A2). After adding the spring-breeding amphibians, we allowed the animals to acclimate for 9 d and then applied the insecticide treatments on 2 June. From this point on, we refer to June 2 as Day 1 of the experiment.

### **2.2.3 Application of pesticide treatment**

All pesticides were purchased as technical grade chemicals (Chem Service, West Chester, PA). On 2 June, to achieve nominal concentrations of 10 ug/L, we added 0.330 ml of a 0.025 g/ml stock solution of chlorpyrifos, 0.339 ml of a 0.023 g/ml stock solution of diazinon, 0.330 ml of a 0.025 stock solution of endosulfan, and 0.343 ml of a 0.024 g/ml stock solution of malathion to the mesocosms. From the same stock solutions, to achieve nominal concentrations

of 40 ug/L, we added 1.32 ml of chlorpyrifos, 1.36 ml of diazinon, 1.32 ml of endosulfan, and 1.37 ml of malathion to the mesocosms. To create the mixture treatment, we combined 0.330 ml of chlorpyrifos, 0.339 ml of diazinon, 0.330 ml of endosulfan, and 0.343 ml of malathion to the mesocosms. For details regarding the confirmation of nominal insecticide concentrations see S.I. 1.3. For simplicity, we will refer to nominal concentration of 10 ug/L as the “low concentration” and 40 ug/L as the “high concentration.”

Approximately 3 wks after adding the insecticides (19 and 23 June), we added two species of summer-breeding amphibians to the mesocosms: gray treefrogs (*Hyla versicolor*) and green frogs (*L. [R.] clamitans*). These tadpoles were selected from a mixture of egg masses and then added to the mesocosms (Table A2). Since summer breeding amphibians were introduced into the mesocosms after the insecticide perturbation, we re-sampled each mesocosm for insecticide concentrations on 20 June (S.I. 1.3).

#### **2.2.4 Abiotic response variables**

On weeks 2, 4, 9, and 18 we quantified temperature, pH, and dissolved oxygen using a calibrated digital water meter (WTW, Woburn, Massachusetts, USA) and quantified light attenuation using an underwater light meter (LI-COR, Lincoln, Nebraska, USA; for additional details see S.I. 1.4).

#### **2.2.5 Biotic response variables**

We sampled zooplankton assemblages during weeks 2, 4, 9, and 18 and identified all zooplankton to the level of species. We then pooled all zooplankton into either cladocerans or copepods. A justification for this decision is provided in S.I. 1.5.



Phytoplankton abundance was measured during weeks 2, 4, 9, and 18 by sampling 500 mL of water from each mesocosm. To assess the abundance of phytoplankton, we measured the concentration of chlorophyll-a in each sample. Additional details can be found in S.I. 1.6.

We measured periphyton abundance by removing a clay tile from each mesocosm during week 2, 4, 9, and 18. The periphyton on the tiles was scrubbed and rinsed with filtered well water. The periphyton-water mix was then filtered through a Whatman GF/C 7-cm filter that had been previously dried at 80° C for  $\geq 24$  hrs. The filters containing periphyton were re-dried for 24 hrs and then re-weighed to determine periphyton biomass.

Snail abundance and diversity was not assessed early in the experiment because the hatchling snails were very small and difficult to accurately assess for abundance. As a result, snail abundance was only assessed at weeks 6 and 18. We did this assessment by counting the number of individuals occupying the sides of the mesocosms from the surface of the water down to a depth of 40 cm (i.e. at the top of the clay tiles that were used as periphyton samplers).

The abundance of detritivores remained low early in the study, so we did not sample their populations until week 13. To assess detritivore abundance, we added mesh bags, containing 15 g of oak leaf litter, to each mesocosm. We first soaked the bags for 3 wks in a wading pool containing natural pond water from three local ponds to allow natural colonization by algae and bacteria. On 14 August, we added the mesh bags to each mesocosm. One week later, we removed one bag from each mesocosm and counted the number of amphipods and isopods.

Over the course of the experiment, the amphibians began to metamorphose. Once the first metamorphs were observed, metamorph emergence was checked daily (for additional details, see S.I. 1.7). We recorded survival to metamorphosis, time to metamorphosis, and mass at metamorphosis. Since green frogs are an overwintering species, they did not undergo

metamorphosis. Therefore, we assessed the individual mass of green frog tadpoles at week 18 by non-destructively sampling five individuals from each mesocosm.

### **2.2.6 Statistical analysis**

Since the data included a large number of response variables that were measured once or more than once during the experiment, we used several different analyses of variance to examine the effects of our treatments. We conducted a repeated-measures, multivariate analysis of variance (rm-MANOVA) on the abiotic response variables that were measured at four time points (temperature, pH, dissolved oxygen, and light attenuation) and on the biotic response variables measured at four time points (cladocerans, copepods, phytoplankton, periphyton). We also used a rm-MANOVA for the snails that were quantified at two time points. For all significant insecticide by time interactions, we used targeted post-hoc tests that separately compared the effect of each insecticide treatment at the four time points. For the two species of detritivores that were measured at a single time point, we analyzed total abundance using an ANOVA and then analyzed amphipod abundance and isopod abundance using a MANOVA. For the five amphibian species that metamorphosed during the experiment, some treatments had no surviving individuals, so these treatments had no mass data. As a result, we conducted separate MANOVAs for survival to metamorphosis, mass at metamorphosis, and time to metamorphosis. The mass of the overwintering green frog tadpole was analyzed separately using an ANOVA.

In all of the analyses, we tested the assumptions and, when necessary, the data were log- or rank-transformed. For all significant ANOVA results, we conducted mean comparison tests using one of three post-hoc tests: Tukey's test for unranked data, S-N-K test for ranked data, and Dunnett's t-test for all comparisons to the control. The effects of the solvent control were never

significantly different from the control, so these data are not reported. All statistical results are presented in the S.I. 2.0- Detailed Results.

## **2.3 RESULTS/DISCUSSION**

The broad goals of this study were to (1) identify the direct and indirect effects of four insecticides on aquatic population and community-level variables, (2) determine what generalizations can be made across insecticide type, organizational levels and time, and (3) investigate whether complex mixtures of insecticides interact in unpredictable, non-additive ways. Towards these goals, we measured >30 separate response variables across 18 wks but in the following sections, we will only be discussing select representative results. All other results are available in S.I. 2.0.

### **2.3.1 Direct effects of individual insecticides on invertebrate populations**

In this section, we will first compare the direct effects of insecticides applied individually on invertebrate population abundance then assess whether traditional measures of toxicity accurately predict population responses and recovery in more complex community settings.

#### **2.3.1.1 Cladoceran response.**

For the three insecticides that shared modes of action (AChE-inhibitor) and chemical class (organophosphate), the low concentration caused different direct effects on cladoceran abundance. In particular, malathion did not cause a reduction in cladoceran abundance but both

diazinon and chlorpyrifos reduced cladoceran abundance at week 2 (Fig. 1; Table A4C). In contrast, when exposed to the high concentration, all three organophosphate insecticides reduced cladoceran abundance at week 2 (Fig. 1). Cladoceran responses to high and low concentrations of these three organophosphates was consistent with past insecticide toxicity estimates (Macek et al. 1976; Vilkas 1976; Naqvi and Hawkins 1989; Cáceres et al. 2007; Chuah et al. 2007).

In contrast, toxicity tests did not correctly predict the effects of the high concentration of endosulfan on cladocerans. Despite the fact that the high concentration of endosulfan used in this experiment was considerably lower than the cladoceran LC50-d values (160 – 3340 ug/L; Chuah et al., 2007; Kegley et al., 2011), we found a significant decline (79%) in cladoceran abundance at week 9 (Fig 1). Relative to traditional short-term measures of toxicity, community studies allow for more complex population responses to insecticides (i.e. sublethal effects of insecticides on time to maturity or reproduction; Newman, 1992). Thus, while the two venues are not directly comparable, the discrepancy between the two study venues, suggests an important area for further investigation. At week 3, endosulfan was not detected in the water; thus, the decline in cladoceran abundance was found after endosulfan had broken down. This suggests that lag effects may be a potential mechanism for the lethal effect of endosulfan on cladocerans. Lag effects of chemical contaminants have previously been documented in several aquatic organisms (Brent and Herricks 1998; Liess 2002; Beketov and Liess 2008). Specific to the insecticide, endosulfan, three recent studies have documented negative lag effects of endosulfan on amphibians (Berrill et al. 1998; Jones et al. 2009; Hammond et al. 2012), but, to our knowledge, this is the first study to suggest such effects on cladocerans. If endosulfan does have negative lag effects on cladoceran populations, it suggests that these lag effects extend across diverse taxa demonstrating the importance of expanding beyond short-term toxicity tests.

### **2.3.1.2 Cladoceran recovery**

When assessing population recovery, two issues we considered were the magnitude of the disturbance's initial effect on a population's abundance (i.e. the population's response) and the persistence of the disturbance. Cladocerans exposed to low concentrations of chlorpyrifos and diazinon both experienced similar initial declines in abundance. However, despite chlorpyrifos persisting in the water longer relative to diazinon (61% chlorpyrifos vs. 30% of diazinon remaining at week 3) cladocerans from both treatments similarly recovered to control abundances by week 18 (Fig 1; Table A4C). Thus, at the lower concentration, the magnitude of the disturbances' initial effect on cladoceran abundance was an accurate predictor of cladoceran recovery and our measures of cladoceran recovery to the low concentration treatment was consistent with the fundamental assumption in toxicology that population recovery can be extrapolated from estimates of individual sensitivities (Newman 2010).

However, we also found that relying solely on the initial magnitude of response can lead to incomplete conclusions about the effects of insecticides on population recovery. For instance, high concentrations of malathion and diazinon both caused similar initial declines in abundance, but cladoceran abundances exposed to malathion recovered by week 9 while those exposed to diazinon did not recover until week 18 (Fig. 1). By week 3, malathion was no longer detectible in the water while 47% of diazinon remained. Thus, the rapid breakdown of malathion relative to diazinon likely contributed to the relatively rapid recovery of cladoceran abundances exposed to malathion versus diazinon. In contrast, persistence did not predict cladoceran recovery to chlorpyrifos. Despite the rapid breakdown of chlorpyrifos in the high concentration (2.8% remaining at week 3), cladoceran abundances exposed to chlorpyrifos did not recover prior to the end of the experiment (Fig. 1). At week 2, not a single cladoceran was detected in the high

chlorpyrifos treatment, which suggests that despite the rapid breakdown of chlorpyrifos, cladoceran abundances were unable to overcome the initial toxicity of chlorpyrifos. One strategy for limiting the damage caused by insecticide contamination is to develop insecticides that break down rapidly (Newman 2010). We demonstrate that the rapid breakdown rates may not necessarily limit the lethal effects of insecticide on populations if the initial toxicity is high.

#### **2.3.1.3 Copepod response**

At the low concentration, chlorpyrifos and diazinon caused a significant decrease in copepod abundance at week 2 but malathion did not (Fig. 1; Table A4C). At the high concentration, malathion and chlorpyrifos caused a decrease in copepod abundance at week 2, but diazinon did not. Unlike cladocerans, copepod abundances were susceptible to both low and high concentrations of endosulfan. Thus, the effects of these chemicals were not generalizable across the two zooplankton groups. Copepods and cladocerans had similar responses to chlorpyrifos and malathion, copepods were more tolerant to diazinon than cladocerans, and copepods were less tolerant to endosulfan than cladocerans. To our knowledge there are no other published toxicity data assessing the response of the species that dominated our copepod assemblages (*Microcyclops rubellus*). However, past work on other species of copepods (*Cyclops* sp.) indicate that our results are generally consistent with past toxicity tests (chlorpyrifos LC502d = 39 µg/L; diazinon LC507d = 2510 µg/L; malathion LC502d = 1300 µg/L; Kegley et al., 2011).

#### **2.3.1.4 Copepod recovery**

Despite similar initial responses, copepod recovery to low concentrations of chlorpyrifos, diazinon, and endosulfan varied. Copepods exposed to the low concentration of chlorpyrifos recovered briefly at week 9 but were significantly reduced again at week 18. In this case, the

persistence of chlorpyrifos in the water column likely contributed to the suppression of cladoceran recovery (61% chlorpyrifos remaining at week 3). In contrast, insecticide persistence did not accurately predict copepod recovery to low concentration of endosulfan and diazinon as both recovered to control abundances at week 4 (Fig. 1; Table A4C) despite the fact that diazinon persisted longer in the water column compared to endosulfan (30% of diazinon and 0% of endosulfan remaining at week 3). While we did not detect malathion and endosulfan in the water column at week 4, it is possible that the concentrations can just be below the limit of detection (0.1 µg/L; S.I. 1.3). At the high concentrations, chlorpyrifos, malathion, and endosulfan all caused a drastic initial decline in copepod abundance (Fig. 1). Copepods exposed to malathion recovered by week 4 but copepods exposed to chlorpyrifos and endosulfan did not recover to control abundances prior to the end of the study. Three weeks after dosing, 0% of endosulfan, 0% of malathion, and 2.7% of chlorpyrifos remained in the water. Thus, similar to cladoceran recovery, despite the rapid degradation of endosulfan and chlorpyrifos, copepod abundances were unable to overcome the initial toxicity of these insecticides.

To sum, traditional toxicology measures of toxicity and insecticide persistence did not always accurately predict copepod recovery indicating that there are likely other factors driving these patterns of copepod recovery when populations are embedded in complex interacting community scenarios. Past studies indicate that in complex community scenarios, pesticide-induced changes in life history or competitive ability can shape patterns of population recovery (Liess et al., 2013, 2006). Thus, future studies incorporating step-wise approaches toward integrating ecological complexity can help identify the factors driving population recovery.

#### **2.3.1.5 Detritivore response**

Despite the enormous diversity of organisms, our understanding of insecticide risk is often biased towards particular model organisms (Kefford et al. 2005). One result of this bias is that we know little about some major functional groups including detritivores in community settings (Hoekman et al. 2009). To address this issue, we incorporated two common detritivores (amphipods and isopods) in our mesocosm community and measured their responses to the insecticide treatments. We found that low concentration of diazinon, malathion, and endosulfan had no effect on amphipods while chlorpyrifos completely eliminated all amphipods (Fig. 3; Table A5C). At the high concentration, amphipod abundances were severely reduced by malathion and completely eliminated by chlorpyrifos, diazinon, and endosulfan. Though only one study has examined the effects of insecticides (diazinon) on the amphipod species used in our study (Arthur et al. 1983), our findings are consistent with published toxicity tests on other amphipod species (Leight and Van Dolah 1999; Anderson and Lydy 2002; Wan et al. 2005; Cothran et al. 2010). For isopods, the only insecticide treatments that negatively impacted population abundances were the low and high concentrations of endosulfan, which completely eliminated all isopods (Fig. 3; Table A5C). These results are consistent with published LC50 values (EPA Supplementary Information, 2010; Kegley et al., 2011; Rubach et al., 2011). Future studies continuing to explore the impact of insecticides on taxa not typically considered in traditional toxicity tests are critical to expand our ability to assess risk for the enormous diversity of organisms potentially exposed to insecticides (Kefford et al. 2005).

#### **2.3.1.6 Snail responses**

The abundance of snails was not affected by any pesticide treatment (Table A6). Past studies also have found that snails are generally tolerant to insecticides (Cuppen et al 2002, Relyea 2005).



Snails play significant roles in aquatic system nutrient cycling (Vanni 2002) thus their ability to tolerate insecticide disturbances may lead to broad scale community-level consequences in highly contaminated systems. Future studies examining the extended consequences of insecticide-induced shift in community diversity are necessary.

### **2.3.2 Indirect effects of individually applied insecticides**

Growing studies demonstrate the importance of moving beyond the population-level to assess the indirect community-level responses caused by insecticides (Fleege et al. 2003; Relyea and Hoverman 2006; Peters et al. 2013). Given the diversity of available insecticides, we need to develop generalizations about the indirect community consequences initiated by different insecticides. We identified a number of indirect effects of insecticides at the community level; in this section, we focus specifically on the generalizability of the indirect effects initiated by the direct effects of the four insecticides on zooplankton response and recovery. Additional indirect effects are discussed in S.I. 2.6.

#### **2.3.2.1 Indirect effects of insecticides on phytoplankton and abiotic variables**

We found that the cascading indirect effects of insecticides were not generalizable across the different insecticides. Instead, the unique direct effects of the different insecticides on cladoceran response and recovery, led to unique indirect cascading effects on other members of community and on abiotic variables. Past studies have demonstrated that declines in cladoceran abundances can facilitate phytoplankton abundances (Downing et al. 2008; Relyea and Diecks 2008). Indeed, we also detected algal blooms (i.e. increased chlorophyll-a concentrations), but only in treatments that significantly suppressed cladoceran recovery (low and high concentrations of

chlorpyrifos and diazinon; Fig. 2; Table A4). We demonstrate that a short-term decrease in cladoceran abundance is not sufficient to induce phytoplankton blooms; only insecticides that both depress abundance and suppress recovery caused the indirect facilitation of phytoplankton abundance. While insecticides that caused a decrease in cladocerans led to increases in phytoplankton abundance, the low concentration of endosulfan caused higher cladoceran abundances (Fig. 1; S.I. 2.6) relative to controls at week 2 and a subsequent a suppression of phytoplankton abundance.

The direct effect of insecticides on the response and recovery of cladoceran populations also had an impact on abiotic variables. In particular, treatments that depressed cladoceran abundance and suppressed recovery also caused associated increases in pH (Fig. A2; Table A3), dissolved oxygen (Fig. A3; Table A3), and light attenuation (Fig. A4; Table A3) which are known to occur during blooms (Relyea and Diecks 2008). Thus, cladoceran population response and recovery are critical to predicting indirect trophic cascades on community biotic variables and the subsequent shifts in abiotic conditions. A challenge in toxicology is to understand the diverse impact of insecticides on the global diversity of organisms (Newman 2010). Considering the response and recovery of taxa to insecticides in complex community scenarios can facilitate our ability to make predictive and mechanistic generalizations about the role of insecticides in shaping patterns of species abundance in natural systems.

### **2.3.3 Direct effects of individual insecticides on spring-breeding amphibians**

A general assumption in toxicology is that by assessing chemical risk and developing regulations based on the responses of the ‘most sensitive’ taxa, less sensitive taxa would inadvertently also be protected (Newman 2010). However, in complex community scenarios, insecticides have

unanticipated indirect lethal consequences on taxa that were assumed to not be sensitive (Relyea and Diecks 2008). Thus, considering ‘less sensitive’ taxa in community scenarios is important to understanding the broad effects of insecticides on aquatic systems. Towards this goal, this section examines the direct consequences of insecticides on amphibians, a relatively more tolerant taxon.

Past toxicity studies indicate that amphibians should be highly tolerant to both high and low concentrations of the organophosphate insecticides used in our study (LC50-d values are 440 to 7,500 ug/L for diazinon; 90 to 3,000 ug/L for chlorpyrifos and 200 to 5,200 ug/L for malathion; Kegley et al., 2011; Lawrence and Isioma, 2010; Sparling and Fellers, 2007). Indeed, when we assessed amphibian survival, we found that a single application of the low concentration treatment only reduced the survival in one case (toads exposed to diazinon; Fig 4; Table A7). At the high concentrations, not a single organophosphate insecticide significantly affected spring-breeding amphibian survival.

The low concentration of endosulfan also had no significant effect on amphibian survival, but the high concentration of endosulfan caused a sharp reduction in the survival of American toads and the complete elimination of wood frogs and leopard frogs (Fig 4). Further, amphibian mortality at the high concentration of endosulfan followed distinct phylogenetic patterns, which is consistent with the conclusion of Hammond et al. (2012): wood frogs, leopard frogs, and American toads are more sensitive (LC50-d = 31, 51, and 56 ug/L, respectively) than spring peepers (LC50-d = 112 ug/L). The three most sensitive species experienced higher mortality than one would predict from these lab LC50 values; 92% of wood frogs, leopard frogs, and American toads were eliminated by the high concentration of endosulfan. This higher than expected amphibian death is likely due to the lag effects that are known to occur with endosulfan

(Berrill et al. 1998; Jones et al. 2009; Hammond et al. 2012). While a growing number of studies has demonstrated delayed effects of contaminants, (Brent and Herricks 1998; Beketov and Liess 2008), our understanding of these lag effects in more realistic community scenarios, is limited (Hammond et al. 2012) and future studies are critical to determine the prevalence of this phenomenon.

#### **2.3.3.1 Role of oviposition phenology on gray treefrog survival**

In addition to lag effects, consideration of amphibian life history, such as variation in oviposition phenology, was also important to understanding amphibian responses to insecticides. For the summer-breeding gray treefrogs, we found no effect of the low concentration of insecticide on survival to metamorphosis. In contrast, at the high concentration, malathion caused a significant reduction in gray treefrog survival (Fig. 4). At week 3 when gray treefrog tadpoles were added to the mesocosm, malathion had completely degraded from the tanks. Thus, these results indicate that the reduction in gray treefrog survival was not due to direct effects of malathion. Instead, this high mortality may be due to the high toxicity of the breakdown product of malathion (maloxon), which is 100 times more toxic to amphibians than the parent compound (Sparling and Fellers 2007). Understanding the temporal overlap between life history characteristics such as oviposition phenology and exposure time to insecticides prior to breakdown are important issues to consider in future studies.

Despite the fact that the initial doses of both low and high endosulfan treatments were higher than their 8-d LC50 value (6 µg/L; Jones et al., 2009), there was no lethal effect of endosulfan on the survival of the summer-breeding gray treefrogs (Fig. 4; Table A7). Again, variation in oviposition phenology played an important role in determining the effects of insecticides on amphibians. While the spring-breeding amphibians experienced high mortality

when exposed to the high concentration of endosulfan, by the time we added summer breeding gray treefrog tadpoles (3 wks after the initial dose), endosulfan had completely broken down in the mesocosms. Past studies indicate that the breakdown product of endosulfan (endosulfan sulfate) tends to be less toxic than the parent compound (Wan et al. 2005; Sparling and Fellers 2009).

Our results suggest that later oviposition phenology negatively affected gray treefrog survival in the malathion treatment, but it facilitated gray treefrog survival in the endosulfan treatment. Thus, consideration of organism phenology, insecticide breakdown rate, and the toxicity of break products is important to identifying potential legacy effects of insecticides. As insecticide use continues to grow, combining our toxicological understanding of chemicals with our ecological understanding of species interactions and life history can facilitate our ability to make predictions about the complex consequences of insecticides natural systems.

#### **2.3.4 Effects of an insecticide mixture**

Despite increasing awareness of insecticide mixtures contaminating aquatic systems (Belden et al. 2007), few studies have considered both the direct and indirect effects of mixtures at the population and community levels. In aquatic mesocosms, (Relyea 2009) found that five insecticides applied as a mixture was significantly more lethal compared to each insecticide applied individually for several taxa. However, one criticism of Relyea (2009) is due to the additive nature of the mixture treatment, mesocosms exposed to the mixture treatment in that study contained five times more insecticide than when each was applied individually. Thus, it is unclear whether the highly lethal results were due to the interaction between the various insecticides or exposure to higher concentrations of insecticide (also see Hayes et al., 2006). To

address this criticism, in addition to comparing the effect of insecticides in mixtures to the additive concentrations of the insecticide applied individually, we also compared the mixture treatment to individual insecticide applications at a concentration equivalent to the mixture (substitutive concentration). Thus, if the effects of the mixture do not exceed that of the substitutive treatment, we can confirm that the effects of mixture relative to the additive concentration are indeed non-additive and not a by-product of having a higher concentration (Hayes et al. 2006; Relyea 2009). No studies to our knowledge have incorporated both the additive and substitutive concentrations to evaluate the effects of insecticide mixture across the populations and communities levels. For a detailed description of our methods for assessing insecticide mixtures, refer to the S.I. 3.0.

#### **2.3.4.1 Direct effects of mixtures at the population level**

For all animal taxa, we first compared the direct effect of the insecticides applied as a mixture to insecticides applied individually at low (additive) concentration treatment. Out of the total 36 cases, we identified 17 cases where insecticides applied in a mixture had significantly more severe consequences compared to individually applied insecticides (Figures 1-6; Table A12; for details regarding these specific cases, refer to S.I. 4.0). In each of these cases, the high (substitutive) concentration treatments of individual insecticides never had a significantly larger impact than the mixture treatment (Table A12). Thus, we can confirm that the severe effects of mixtures were not due to the higher total concentration of the mixture treatment. When applied in combination, pesticides have been documented to have unanticipated synergistic effects (i.e. response to the insecticide mixture is greater than the response of insecticides applied individually; Tabashnik, 1989). We show that these lethal effects of mixtures are also pervasive across a wide range of interacting aquatic taxa. Pesticides have also been documented to have

antagonistic effects (i.e. responses to insecticide mixtures are less severe than responses to insecticides applied individually; Darling and Côté, 2008). In this study we did not detect antagonistic effects but we only considered one type of mixture. Given the prevalence of insecticide mixtures in aquatic systems, an important challenge will be to develop generalizations about when mixtures lead to additive, synergistic, or antagonistic effects.

#### **2.3.4.2 Indirect effects of mixtures**

Similar to the individually applied insecticides, the direct mortality and suppression of cladoceran abundances by the mixture treatment indirectly facilitated phytoplankton abundance (Fig. 2; Table A4) and led to an increase in pH (Fig. A2; Table A3). Additionally, the mixtures caused a significant reduction (75% mortality) across all spring-breeding amphibian species (Fig 5; Table A7). Specifically, the lethal direct effects of the insecticide mixture on wood frogs, spring peepers, and leopard frogs led to decreased overall tadpole competition for resources as evidenced by the large amounts of filamentous algae that we observed floating in these mesocosms (J. Hua and R.A. Relyea, personal observation). This increase in resources led to an indirect positive effect on the biomass of surviving amphibians. Surviving spring peepers and leopard frogs from the mixture treatment were 99% and 166% heavier than those in the control. Further, leopard frogs from the mixture treatment were 53%, 60%, and 68% heavier than leopard frogs in the low concentrations of chlorpyrifos, diazinon, and malathion, respectively (Fig. 5; Table A8). Similarly, peepers exposed to mixtures were 39% and 48% heavier than peepers in the low concentration of malathion and endosulfan, respectively. Thus, mortality in the spring breeding species caused by mixtures led to decreased competition, ultimately increasing food sources for surviving spring amphibians and facilitating growth (Relyea 2009).

In addition to facilitating the surviving spring-breeding species, we found that the lethal effect of mixtures on spring-breeding species also benefitted summer-breeding amphibians, which were added three weeks after the insecticides were applied. Gray treefrogs exposed to the mixture were 38%, 60%, 37%, 30% and 59% heavier than gray treefrogs in the control and the low concentrations of malathion, diazinon, endosulfan, and malathion respectively (Fig. 5). In addition, compared to the control, gray treefrogs exposed to mixtures not only emerged heavier but they completed metamorphosis faster (Fig A5; Table A9). Given the fast breakdown rate of insecticides, by the time summer breeding species entered the experiment, the majority of insecticides had broken down. Though these amphibians were never directly exposed to insecticides, there was a legacy of past insecticide history on their biomass. We found that that the legacy of insecticides positively impacted the biomass of these gray treefrogs; however, it is important to note that the long-term effects are unknown. As metamorphosing amphibians leave their natal ponds, they contribute to a significant loss of energy that may have lasting effects on the future community productivity (Smith 1987; Altwegg and Reyer 2003). Our results indicate that insecticides can have unexpected positive consequences even once they are gone, but future studies long-term are necessary to fully understand these overall consequences.

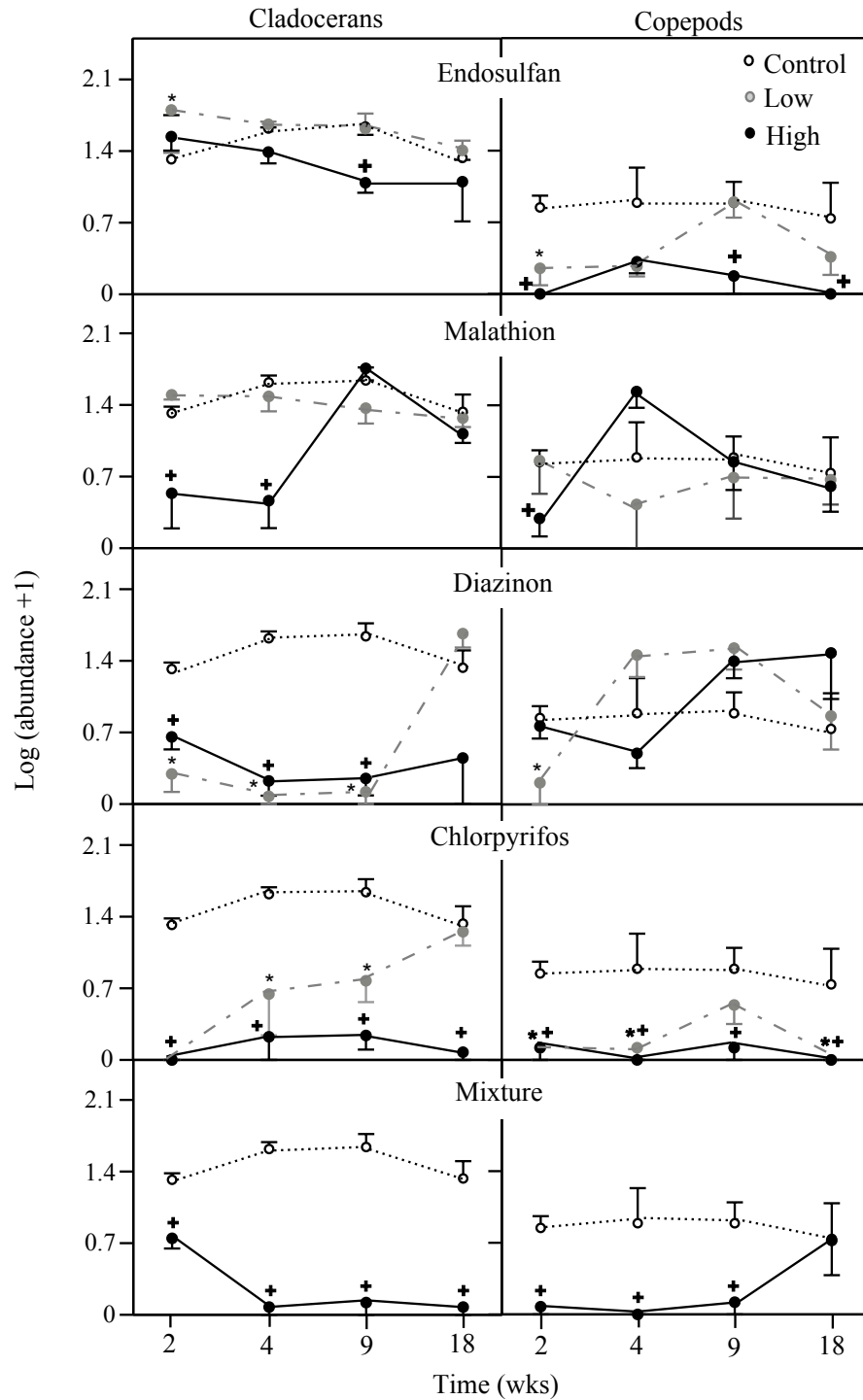
## **2.4 CONCLUSIONS**

Responses to different insecticides, even insecticides that share chemical properties, can vary dramatically. We demonstrate that the direct effects of the different insecticides on zooplankton had unique indirect effects on competing zooplankton abundances and cascading indirect consequences on algal abundance and abiotic variables. Additionally, though most insecticides



are meant to work quickly and then degrade, they can continue to impact natural systems across time. Collectively, these results highlight the importance of considering multiple organizational levels and time in future assessments of insecticide toxicity.

Finally, aquatic systems commonly face complex mixture of insecticides. Compared to insecticides applied individually, insecticides in mixture can have unanticipated positive and negative direct consequences that can lead to indirect consequences at the community level and alter abiotic variables. We examine only one mixture combination— future studies should examine other mixtures with the ultimate goal of developing our ability to predict situations in which additive, synergistic, or antagonistic scenarios of mixtures occur.



**Figure 2-1 Effects of four insecticides, applied separately and in a mixture, on the abundance of cladocerans and copepods across time. Data are mean  $\pm$  SE. The symbols \* and + indicate times at which the low and high treatments, respectively, differed from the control  $p < 0.05$ .**

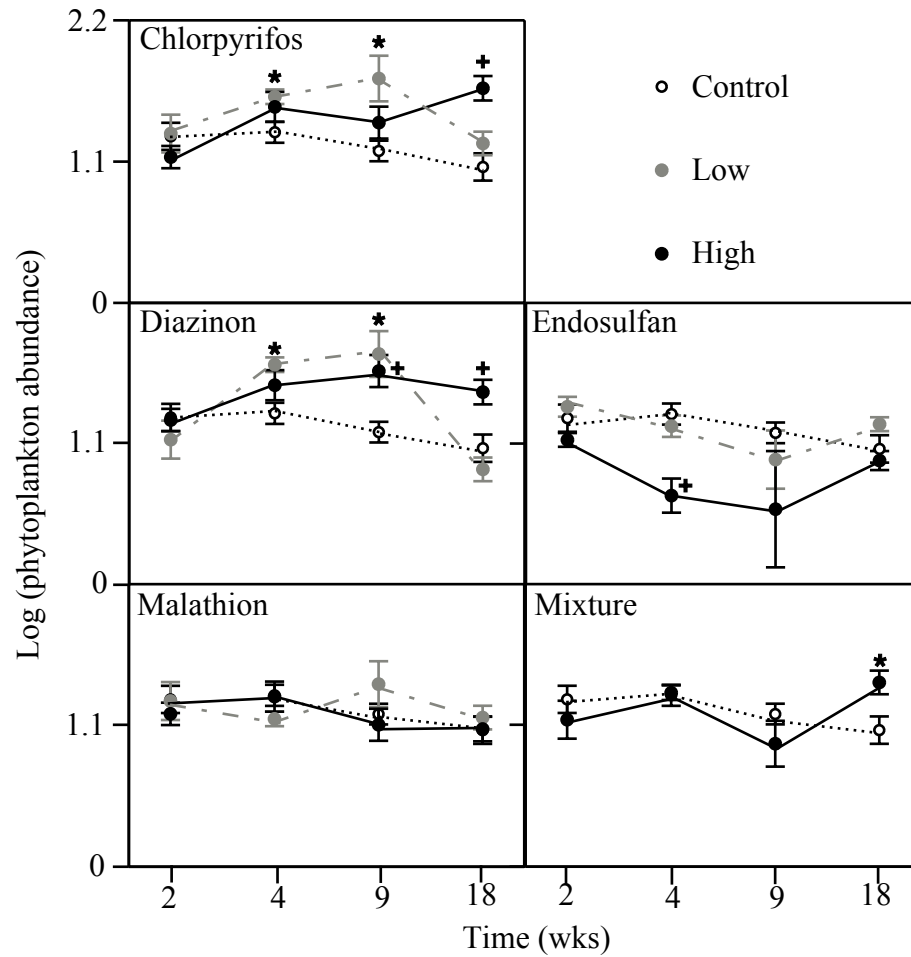
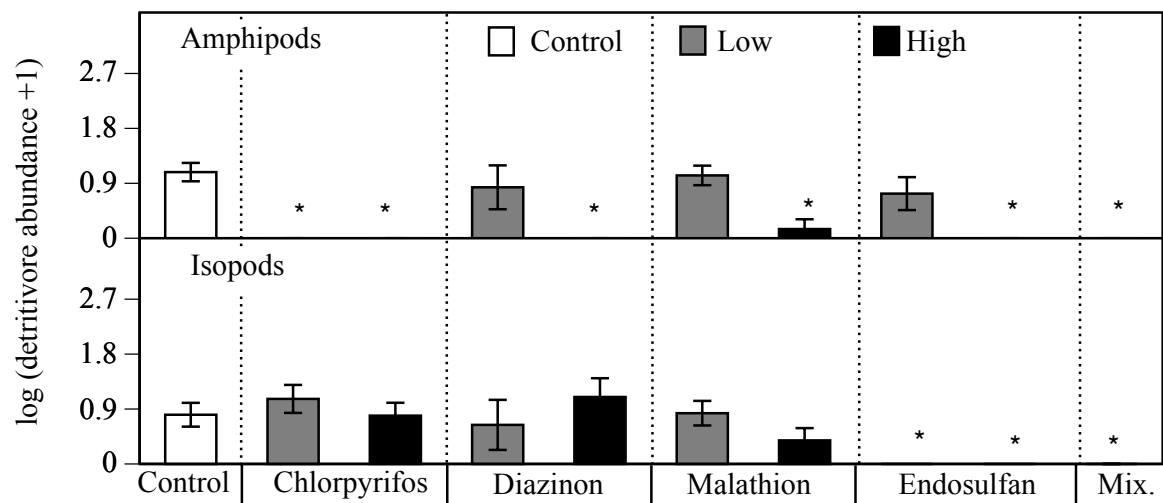
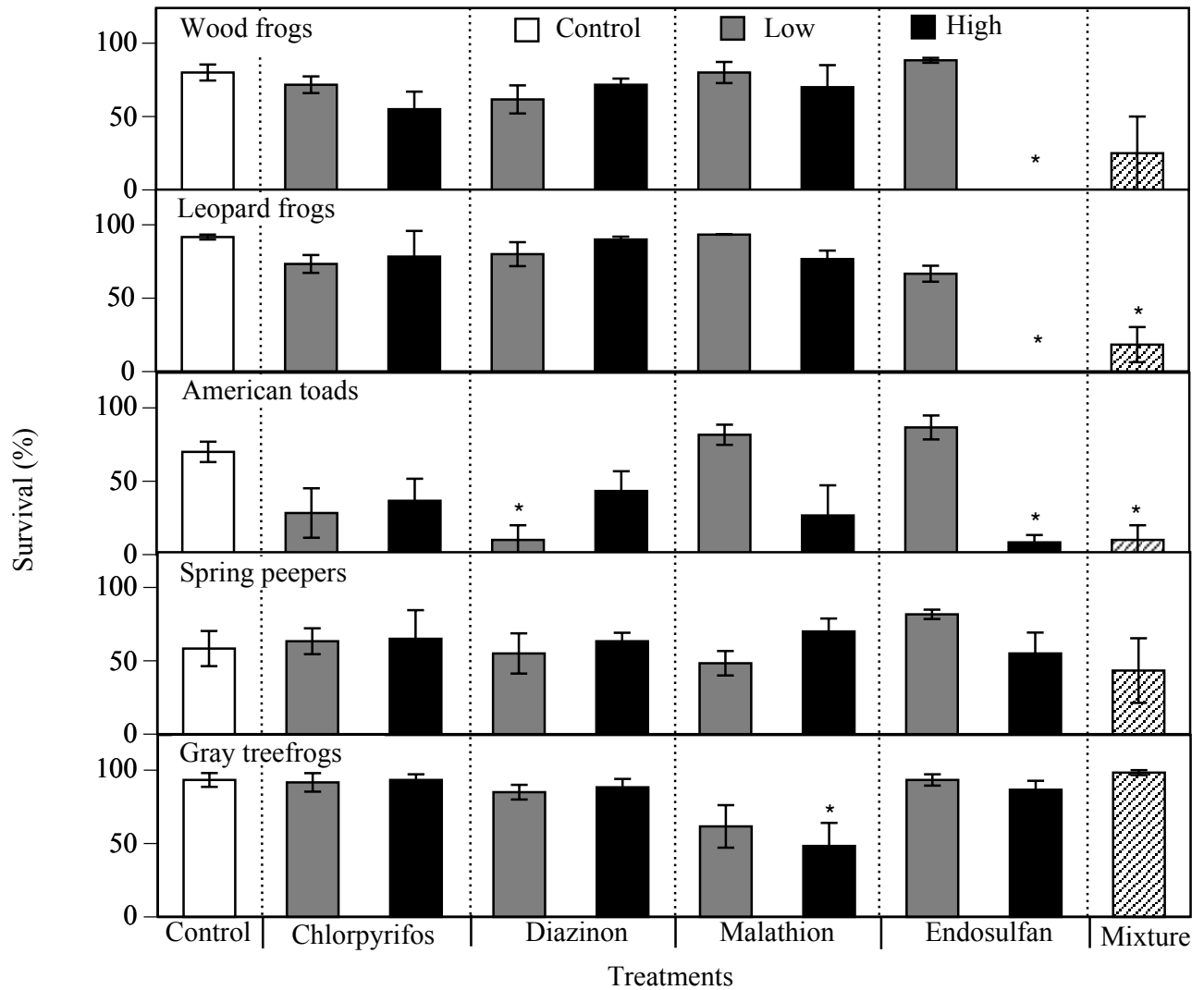


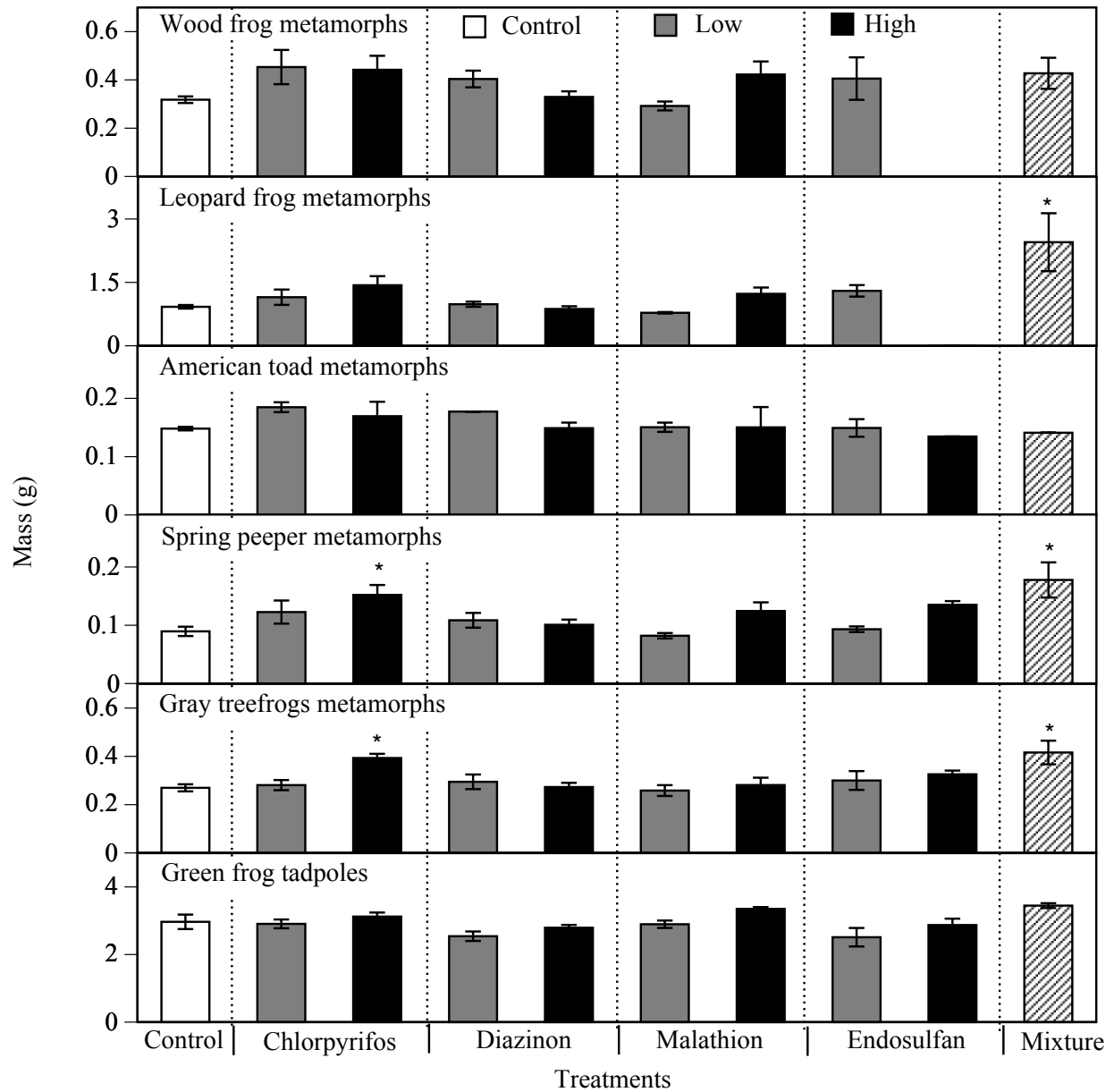
Figure 2-2 Effects of four insecticides, applied separately and and in a mixture, on the abundance of phytoplankton over time. Data are means  $\pm$  SE. The symbols \* and + indicate times at which the low and high treatments, respectively, differed from the control  $p < 0.05$ .



**Figure 2-3 Effects of four insecticides, applied separately and in a mixture, on the abundance of amphipods and isopods at week 13. Data are means  $\pm$  SE. Asterisks indicate treatments that differ from the control  $p < 0.05$ .**



**Figure 2-4** Effects of four insecticides, applied separately and in a mixture, on the survival of the five metamorphosing amphibians. Data are mean  $\pm$  SE. Asterisks indicate treatments that differ from the control  $p < 0.05$ .



**Figure 2-5 Effects of four insecticides, applied separately and in a mixture, on wood frog, American toad, spring peeper, leopard frog, and gray tree frog biomass at metamorphosis and green frog tadpole mass. Data are mean  $\pm$  SE. Asterisks indicate treatments that differ from the control  $p < 0.05$ .**

### **3.0 CROSS-TOLERANCE IN AMPHIBIANS: WOOD FROG MORTALITY WHEN EXPOSED TO THREE INSECTICIDES WITH A COMMON MODE OF ACTION**

#### **3.1 INTRODUCTION**

The evolution of pesticide resistance in target species has become a severe problem costing over \$1.5 billion each year (Georghiou 1990; Pimentel 2005). Pesticide tolerance is particularly costly when it confers increased survival to organisms across different pesticides (known as cross-tolerance; (Dunley and Welter 2000). Cross-tolerance is frequently observed in pest species and most commonly among chemicals with similar modes of action, although cross-tolerance can also occur among pesticides with different modes of action (Alou et al. 2010). Despite the evidence for cross-tolerance in pest species, cross-tolerance and its implications for non-target organisms have been largely overlooked (but see (Brausch and Smith 2009).

The possibility of cross-tolerance in non-target species has substantial ecological and conservation implications. Pesticides can impose strong selection that affects both the traits involved in conferring tolerance as well as correlated traits. Due to pleiotropic effects, pesticide tolerance may carry a fitness cost that will reduce the health of populations after exposure (Hoffmann and Parsons 1991; Semlitsch et al. 2000). Additionally, pesticide-imposed selection can reduce the genetic diversity of populations, which may hamper their ability to respond to changing environments (i.e. the multiple stressor hypothesis; see (Jansen et al. 2011).

Cross-tolerance to pesticides may dampen these negative effects of pesticide-imposed selection by reducing the episodes of selection experienced by populations. Thus, investigating cross-tolerance is important for understanding the ecological and conservation ramifications of pesticide inputs into natural systems.

Small ponds and pools provide excellent systems to study patterns of pesticide tolerance in non-target species. These systems are abundant, have well-defined populations of organisms and receive variable amounts of anthropogenic stress including insecticides (De Meester et al. 2005; Gilliom 2007). Furthermore, ponds and pools are the preferred habitat of many amphibians, which are experiencing worldwide population declines for a variety of hypothesized reasons including exposure to insecticides (Sparling et al. 2001; Wake 2012). Amphibian populations are often exposed to a number of insecticides that vary in their chemical and toxic properties (Faust 2000). Moreover, amphibian tolerance to pesticides can be highly enigmatic with tremendous variation across amphibian families (Jones et al. 2009), among species within families, and among populations (Bridges and Semlitsch 2000). This population variation in tolerance allows for the unique opportunity to investigate whether populations that are tolerant to a particular insecticide are also tolerant to other insecticides.

Given the number of available pesticides and their diverse effects across amphibians, determining general trends of tolerance of even the most commonly used pesticides can be a formidable task. However, by grouping pesticides by their modes of action, it is possible to generalize the effects of multiple pesticides (Clements and Rohr 2009). Acetylcholine esterase (AChE)-inhibiting insecticides (e.g., carbaryl, malathion, and chlorpyrifos) are a group of pesticides that are often used to control pest insects (Grube et al. 2011). These insecticides function by reversibly or irreversibly binding to AChE. With AChE inhibited, acetylcholine



accumulates causing an overstimulation of neurons and eventually mortality (Brown 2005; <http://www.entmclasses.umd.edu/peap/leaflets/pil43.pdf>). Due to the broad similarity in function across insecticides in this group, mechanisms conferring tolerance are likely to be similar. To our knowledge, the phenomenon of cross-tolerance to insecticides among amphibian populations has not been investigated.

We investigated whether amphibian larvae show cross-tolerance to insecticides that have the same mode of action. We did this by testing the tolerance of 15 populations of wood frog (*Lithobates* [formerly *Rana*] *sylvatica*) to three commonly used AChE inhibiting insecticides (carbaryl, chlorpyrifos, and malathion) and then determining whether population patterns of tolerance were correlated among the three insecticides.

## **3.2 METHODS**

### **3.2.1 Pesticide background**

We used three commonly applied insecticides: carbaryl, chlorpyrifos, and malathion. These insecticides vary in their toxicity and chemical classes, but share the same mode of action. Chlorpyrifos and malathion are organophosphates whereas carbaryl is a carbamate. All three of these insecticides kill organisms by inhibiting AChE activity and are used in the agricultural sector as well as for residential and public pest-control. Moreover, all three are found in aquatic systems due to direct application, drift, and run-off [though lower concentrations are more common, maximum concentrations detected in nature in ppb: carbaryl = 2500, chlorpyrifos =

5.8, and malathion = 583; California Department of Fish and Game, 1982; Norris et al. 1983; Kozlowski et al. 2004).

### **3.2.2 Animal collection and husbandry**

Animals were collected as early-stage embryos (Gosner stage 10-12; 10 clutches from each population) from 15 ponds across western Pennsylvania, USA (Supplemental data, Table S1; Gosner 1960). Wood frogs typically move less than 300 m from their natal pond and the genetic neighborhood is generally within ~ 1 km of the breeding pond (Berven and Grudzien 1990; Semlitsch 1998; Semlitsch 2000). The two closest ponds in our study are separated by 4 km, thus it is very unlikely that frogs collected from different ponds were from the same population. All clutches were collected within a 7-d period. To avoid the confounding effects of developmental stage and size on sensitivity to insecticides, we manipulated temperature to standardize hatching time (Pearman and Garner 2005). Clutches collected prior to 11 April were raised outdoors in 100-L pools filled with 90 L of aged well water (air temperature ranged from -1°C to 27 °C). Clutches collected on 11 April were initially held indoors in 14-L plastic containers filled with 10 L of filtered water at a constant temperature of 20°C for 3 d. These indoor temperatures were warm enough to allow for faster embryonic development, but are within the range of temperatures experienced by tadpoles in nature. When developmental stages of all clutches had converged, they were transferred to 100-L pools and development continued outdoors. All 15 populations hatched within a 20-h period. To avoid density-dependent variation during the initial stages of larval development, we transferred 300 tadpoles from each population (Gosner stage 25; a safe tadpole handling stage) to common garden pools (100-L pools filled with 90 L of aged well water) and fed them 5 g of rabbit chow weekly until used in the experiment (~ 2 wks).

Wood frog mass ranged from  $0.5 \pm 0.1 - 0.8 \pm 0.2$  g and did not differ across the 15 different populations at the start of the experiment ( $F_{14, 150} = 0.647$ ,  $P = 0.822$ ).

### **3.2.3 Cross-tolerance experiment**

We used a completely randomized, factorial design with 15 populations of *L. sylvatica* crossed with four insecticide treatments (water control, 6 ppm of carbaryl, 1.75 ppm chlorpyrifos, or 10 ppm malathion). We chose these concentrations of insecticides based on pilot studies; though these concentrations are unrealistically high relative to what is generally found in nature, our objective was to ensure that the tadpoles experienced some mortality to each insecticide so that we could determine if population tolerance was correlated across the three insecticides. To make insecticide solutions, we first prepared stock solutions by diluting technical grade chemicals (purchased from Chem Service, West Chester, PA, USA) in ethanol (stock solution concentrations in ppm: carbaryl = 5000, chlorpyrifos = 2024, malathion = 5000). We then prepared working solutions of each insecticide by adding 7.8, 5.8, or 13  $\mu$ L of stock solution to 6.5 L of carbon-filtered, UV irradiated water for carbaryl, chlorpyrifos, and malathion, respectively. Samples (500 mL) of each working solution were sent to an independent laboratory (Mississippi State Chemical Laboratory, Mississippi State, MS, USA) to ascertain actual concentrations for each insecticide. Actual concentrations were 1.9, 0.3, and 0.4 ppm for carbaryl, chlorpyrifos, and malathion, respectively. Samples were assessed three months after the experiment, thus though they were stored according to established analytical methods (Sherma and Beroza 1980) it is likely that the samples experienced some degradation prior to testing (Maštovská and Lehotay 2004; OECD 2007). Solvent (ETOH) concentrations in working solutions were below the ASTM solvent standard (0.1 mL/L ETOH) for aquatic test species

(ETOH concentrations in our working solutions: carbaryl = 1.2  $\mu\text{L/L}$ , chlorpyrifos = 0.9  $\mu\text{L/L}$ , and malathion = 2  $\mu\text{L/L}$ ). We chose not to incorporate a solvent control since past studies have demonstrated that even higher solvent concentrations do not decrease tadpole survival (Jones et al. 2009).

Experimental units were Petri dishes (100 mm diameter-by-20 mm height) filled with 70 mL of control or insecticide solution and 10 tadpoles. The 60 treatments were replicated five times for a total of 300 experimental units. We assessed tadpole mortality every 4 h over an 80-h period and, at each assessment, we removed the dead tadpoles from dishes. We determined death by gently prodding tadpoles with a pipette and looking for movement. From these data, we calculated time-to-death (TTD) for tadpoles exposed to insecticides. Control survival was high at 99% survival; thus we did not calculate TTD for the controls. For the 42% of the tadpoles that remained alive in the insecticide treatment after 80-h, we assigned them a TTD value of 80 h. Of the 42% of tadpoles that survived, 17%, 40%, and 43% were exposed to carbaryl, malathion, and chlorpyrifos, respectively. These static systems were renewed with treatment water every 24 h. In accordance with standard toxicity protocols, tadpoles were not fed during the experiment. All work was approved by the University of Pittsburgh's Institution Animal Use and Care Committee (Protocol #12050451).

#### **3.2.4 Statistical analysis**

We used generalized linear mixed models (GENLINMIXED in SPSS) to determine whether populations, insecticide treatments and their interaction affected TTD of tadpoles. If a population-by-insecticide interaction was found, we used separate mixed models to assess whether populations differed in tolerance within each insecticide. Petri dish was included in all

models as a random effect to account for the fact that individuals within a dish are not independent. We also used a gamma distribution with a log link function to account for the right skew in the data. A sequential Bonferroni adjustment was used to control alpha for pairwise comparisons. To test for cross-tolerance, we used a Pearson's correlation test of the mean population TTD of tadpoles from the insecticide treatments. A one-tailed test was used because we had an a priori expectation that the correlation would be positive due to the insecticides sharing the same mode of action.

### 3.3 RESULTS

We found a significant effect of insecticide treatment ( $F_{2,2202} = 40$ ;  $P < 0.001$ ), population ( $F_{14,2202} = 7.5$ ;  $P < 0.001$ ), and an insecticide-by-population interaction ( $F_{28,2202} = 1.9$ ;  $P = 0.003$ ), on tadpole TTD. Since there was an insecticide-by-population interaction, we conducted separate mixed models to assess population-level variation in tolerance for each insecticide. Populations varied greatly in their tolerance to carbaryl ( $F_{14,735} = 5.7$ ;  $P < 0.001$ ), chlorpyrifos ( $F_{14,735} = 2.5$ ;  $P = 0.002$ ), and malathion ( $F_{14,732} = 12.6$ ;  $P < 0.001$ ; Fig. 1). Tadpole TTD ranged from  $58.1 \pm 3.2$  to  $78.4 \pm 2.3$  h (mean  $\pm$  SE),  $66.9 \pm 8.7$  to  $80 \pm 2.3$  h, and  $68.86 \pm 6.4$  to  $80 \pm 2.3$  h for carbaryl, chlorpyrifos, and malathion, respectively. In summary, there was population-level variation in sensitivity to the insecticides.

### 3.3.1 Cross-tolerance patterns

Tadpole TTD was positively correlated for two of the three pairwise combinations of insecticides (Fig. 2). Tadpole TTD when exposed to carbaryl was positively correlated with TTD when exposed to chlorpyrifos across populations ( $r = 0.56$ ,  $p = 0.015$ ). Similarly, TTD of tadpoles exposed to carbaryl and malathion were positively correlated ( $r = 0.64$ ,  $p = 0.005$ ). In the third comparison, the TTD correlation of tadpoles exposed to malathion and chlorpyrifos was also positive but the correlation was not quite significant ( $r = 0.36$ ,  $p = 0.09$ ).

## 3.4 DISCUSSION

Cross-tolerance to multiple insecticides is hypothesized to be prevalent when insecticides share a similar mode of action (Georghiou 1990), yet there are few examples of cross-tolerance in non-target species (Brausch and Smith 2009) and no examples in amphibians. We found that wood frog populations varied in their sensitivity to three commonly used insecticides and that population-level patterns of tolerance were correlated between carbaryl and both chlorpyrifos and malathion. Although chlorpyrifos and malathion are both organophosphates with the same mode of action, the correlation in tolerance between these two insecticides was not quite significant.

Despite sharing similar modes of action (AChE inhibitor), the degree of cross-tolerance varied between carbaryl, chlorpyrifos, and malathion. In pest species, tolerance to AChE inhibiting insecticides can develop through genetic mutations, metabolic modifications, or behavioral changes. Specifically, mutations that alter AChE target site or metabolic

modifications that up-regulate AChE are commonly associated with insect tolerance to carbamates and organophosphates (Georghiou 1990; Mutero et al. 1994). Insecticides of the same modes of action can have unique consequences on different organisms (Oakeshott et al. 2005) thus, to a better understand cross-tolerance in non-target organisms, a critical challenge for toxicologists is explore the mechanisms driving these varying effects.

Cross-tolerance should increase the likelihood that amphibian populations that are tolerant to one insecticide will be able to survive subsequent exposures to other insecticides that have the same mode of action. This is important because amphibians can be exposed to a wide range of insecticides during their development with each insecticide potentially causing selection for tolerance (Bridges and Semlitsch 2000). Cross-tolerance to insecticides should reduce the episodes of selection experienced by populations and thus minimize both negative pleiotropic effects and eliminate the erosion of genetic variation in the population (Coors et al. 2009; Brévault et al. 2011). In terms of amphibian conservation, our results suggest that amphibians with cross-tolerance are not only more tolerant to the insecticide causing selection, but may also be tolerant to a wide range of similarly acting insecticides. With amphibians worldwide experiencing unprecedented declines and some of these declines being associated with insecticide use (Davidson 2004), quantifying the existence and prevalence of cross-tolerance may contribute important insights towards conservation efforts.

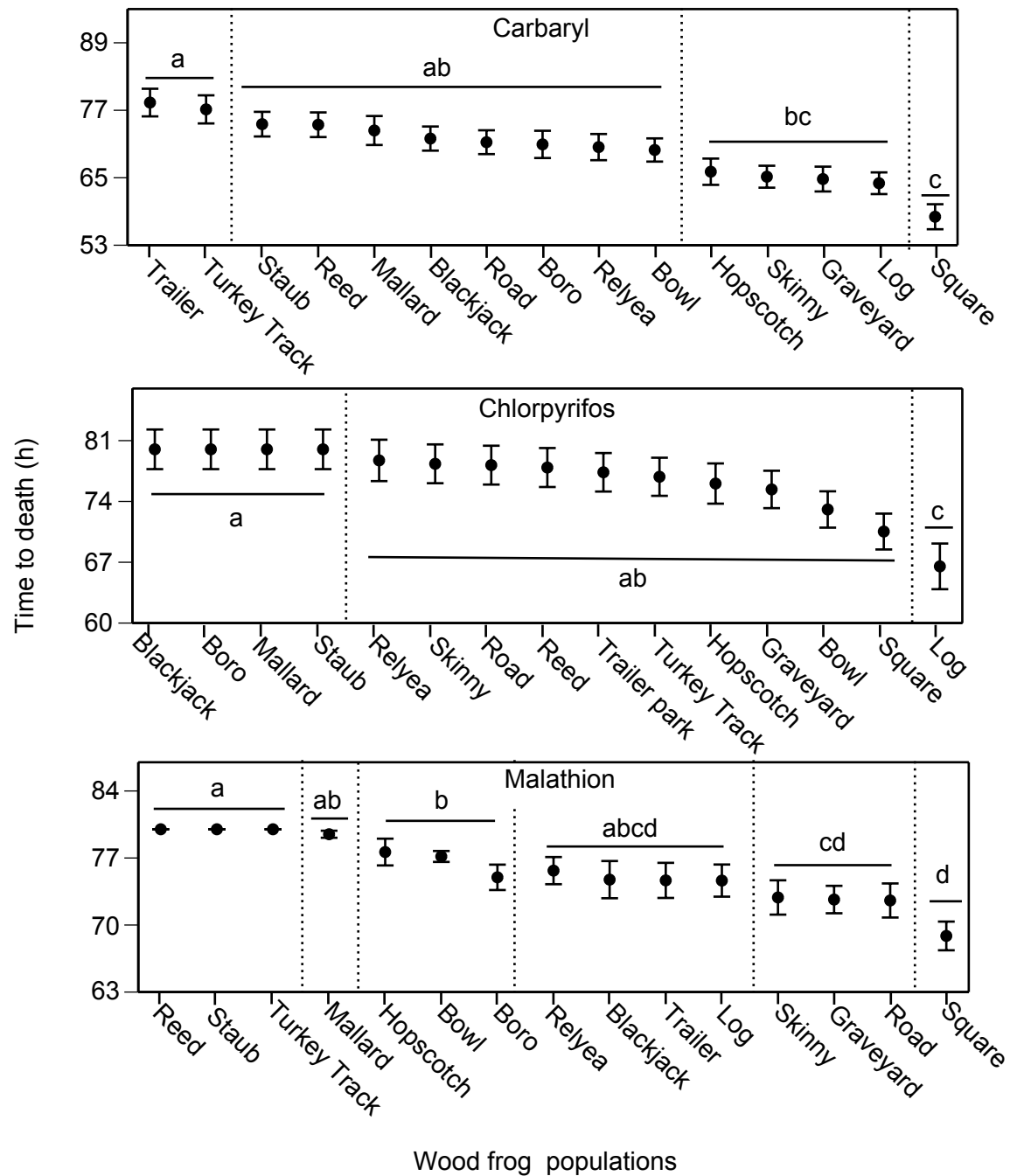
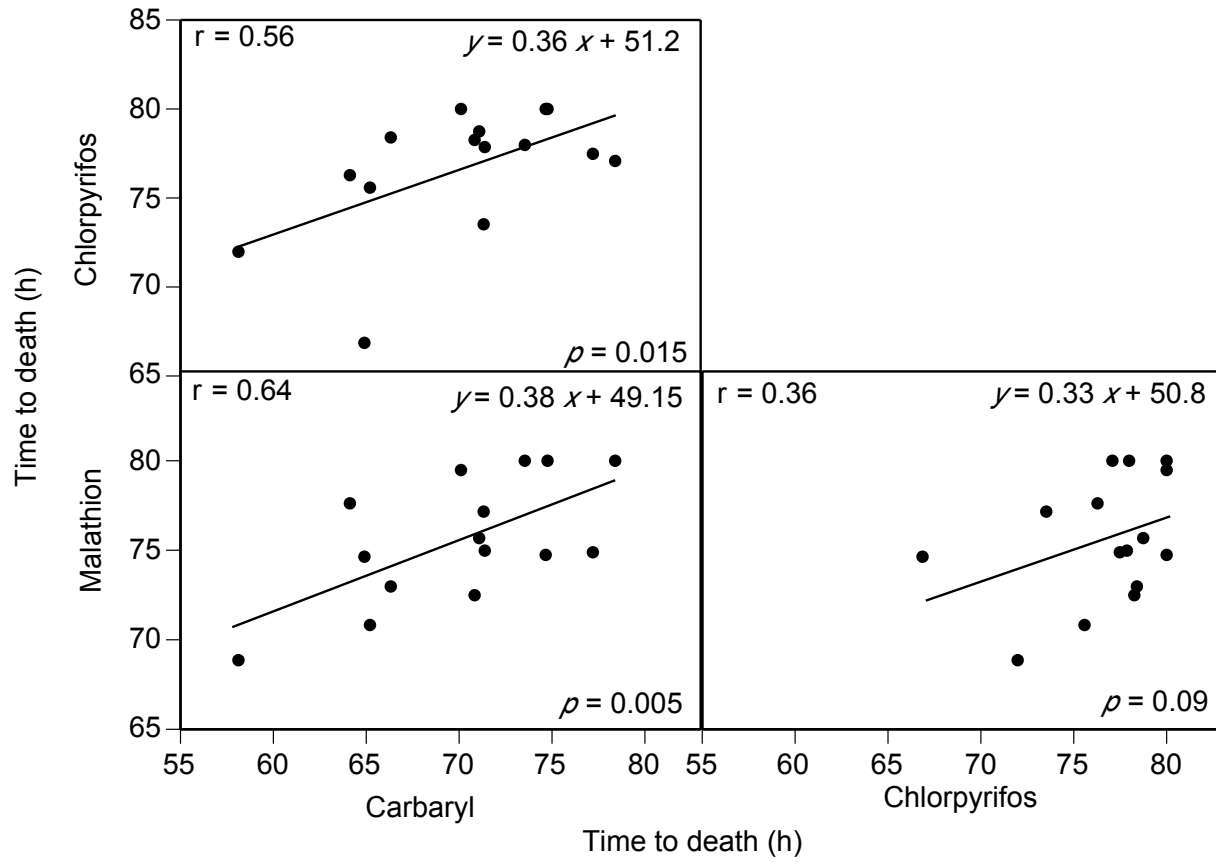


Figure 3-1. Effects of carbaryl, chlorpyrifos, and malathion on tadpole time to death. Data are means  $\pm$  1 SE. Populations sharing similar letters did not differ in sensitivity to insecticides based on Tukey's pairwise comparisons ( $p > 0.05$ ).





**Figure 3-2. Tadpole time to death correlations across carbaryl, chlorpyrifos, and malathion for 15 wood frog populations.**

## **4.0 PESTICIDE TOLERANCE IN AMPHIBIANS: INDUCED TOLERANCE IN SUSCEPTIBLE POPULATIONS, CONSTITUTIVE TOLERANCE IN TOLERANT POPULATIONS**

### **4.1 INTRODUCTION**

Phenotypic plasticity, which is the capacity of a single genotype to exhibit variable phenotypes in different environments, allows adaptive traits to be induced within a single generation (Schlichting and Pigliucci 1998). In rapidly changing environmental conditions, the contribution of plasticity to the process of organismal adaptation has critical implications for the expression of traits within individuals and the evolution of populations. With increasing human influences on the environment, one important challenge is to consider the role of plasticity in shaping organism responses to anthropogenic disturbances.

Chemical contaminants such as pesticides are widely applied in the environment to control pests and prevent human diseases. However, the evolution of pesticide resistance has become a major problem in controlling pest populations and today more than 500 pest species have evolved resistance to various chemicals (Georghiou 1990, Georghiou and Lagunes-Tejeda 1991). Considerable effort has sought to understand the mechanisms behind insecticide resistance, but this understanding has almost exclusively focused on targeted pest species (Jansen

et al. 2011) and is strongly biased towards the assumption that resistance takes the form of a constitutive trait (Feyereisen 1995, Pimental 2005; Lopes et al. 2008).

In contrast, the potential role of plasticity in shaping insecticide resistance (i.e. induced tolerance<sup>1</sup>) has rarely been considered. To our knowledge, only one other study has documented the induction of higher tolerance following prior exposure to pesticides. In that study, Poupardin et al. (2008) found that mosquitoes (*Aedes aegypti*) exposed to different contaminants could induce higher tolerance to several other contaminants, similar to how organisms exposed to predator cues subsequently become less susceptible to predators (Ferrari and Chivers 2009; Schoeppner and Relyea 2009). Given that exposure to insecticides can vary dramatically across space and time (Odenkirchen and Wente 2007), it seems reasonable that targeted pest species and non-targeted species may be able to respond to sublethal exposures by inducing higher tolerance to later exposures. If so, then induced tolerance could play a significant role in population persistence following an initial insecticide exposure. If inducible tolerance to pesticides occurs widely among taxa, it would alter our entire perspective on how pesticides affect organisms in nature.

There are three factors that could potentially affect the magnitude of induced insecticide tolerance: the concentration of the exposure, the timing of the exposure, and the history of a population's exposure. The concentration of the initial sublethal exposure should matter because it may affect an organism's ability to detoxify the insecticide. At low concentrations, researchers have shown that populations have evolved higher resistance by upregulating the enzymes that pesticides target, such as acetylcholine esterase (AChE; Georghiou 1990; Oakeshott et al. 2005).

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<sup>1</sup>We note that the distinction between the terms resistance and tolerance can vary across disciplines. Many toxicologists define tolerance as differences in susceptibility among species or life stages and resistance as the microevolution of decreased susceptibility over time. In this study, we focus on assessing variation in tolerance.

At higher concentrations, however, pesticides may overwhelm the ability for organisms to upregulate these enzymes or the cost of upregulation may simply be too high; if so, then we would not expect to observe induced tolerance at high pesticide concentrations. Given these predictions, it is important to examine the potential for induced tolerance across a range of concentrations.

The timing of the initial sublethal exposure during development should also affect induced tolerance. Toxicology research has found that lethal susceptibility to insecticides can vary with developmental stage (Bridges 2000; Aydın and Köprücü 2005; Jones et al. 2010) and plasticity research has found that developmental constraints (physiological, mechanical, energetic, etc) commonly hinder an individual's ability to initiate plastic responses (Hensley 1993, Hoverman and Relyea 2007). Given the known importance of developmental windows of sensitivity to pesticides, examining the potential for induced tolerance in multiple developmental stages is critical.

The history of a population's exposure to pesticides is another factor that could affect induced tolerance. Plasticity theory predicts that when phenotypically plastic populations experience consistent inducing conditions over multiple generations, a plastic response can eventually become constitutively expressed, even in the absence of original cues that induced the phenotypic change (i.e. the process of genetic assimilation; Waddington 1942, Debat and David 2001). In contrast, when populations experience inconsistent conditions, inducible traits should be maintained because they facilitate the expression of adaptive phenotypes in response to variable environmental conditions (Schlichting and Pigliucci 1998). Applying this theory to induced tolerance, one would predict that induced tolerance would be more likely in populations that are not consistently exposed to insecticides whereas constitutive tolerance would be more

likely in populations that are consistently exposed to insecticides. Given these predictions, it is important to look for patterns of induced tolerance in multiple populations that differ in the regularity of their exposure to insecticides.

#### **4.1.1 Model system**

Amphibians are an excellent model system to investigate the potential for inducible insecticide tolerance. Extensive research has demonstrated the ability of amphibians to alter their behavioral and morphological phenotypes in response to various stressors including competitors, predators and pesticides (Relyea 2001, 2002, Van Buskirk 2009, Relyea 2012). Thus, amphibians may be able to respond to sublethal insecticide exposures, so it is reasonable to investigate whether insecticides can induce increased tolerance.

Given their well-described developmental stages (Gosner 1960), amphibians are also good models for investigating how exposure across ontogeny might affect the inducibility of tolerance. Previous work has shown that amphibian tolerance to insecticides varies across ontogeny, with embryos generally being more tolerant than tadpoles (Bridges 2000). The ability for amphibians to induce tolerance to stressors during more tolerant life stages can have significant lasting consequences on more susceptible life stages by decreasing the lag time between the exposure to a stressor and the ability to mount adaptive responses (Ferrari and Chivers 2009).

Amphibians are also an ideal system to investigate how the proximity of populations to agriculture might impact the ability for individuals to induce tolerance. For example, ponds < 200 m from agriculture are not strongly affected by insecticides (Declerck et al. 2006), which means that amphibian populations that are closer to agricultural fields may be more commonly

exposed to pesticides. In support of this pattern, Cothran et al. (in press) recently discovered that wood frog (*Rana sylvatica* [*Lithobates sylvaticus*]) populations living closer to agriculture are more tolerant to insecticides than populations living far from agriculture. Thus, it is likely that proximity to agriculture can contribute to shaping insecticide tolerance, and the distance from agriculture can provide a unique opportunity to investigate how amphibian populations exposed to different levels of agricultural activities including pesticide applications have evolved different magnitudes of induced tolerance.

Using larval wood frogs, our goal was to determine how different sublethal insecticide concentrations, different times of exposure in ontogeny (embryo and hatchling), and different populations close to and far from agriculture affect induced tolerance in wood frog tadpoles. Specifically, we tested these hypotheses: 1) exposure to sublethal pesticide concentrations will induce higher tolerance to the insecticide later in life, 2) induced tolerance is more likely to occur when individuals are exposed to lower than higher sublethal concentrations, 3) induced tolerance will occur in both the embryos and newly hatched tadpoles, 4) populations located far from agricultural fields will exhibit greater induced tolerance than populations located close to agricultural fields 5) tadpoles with induced tolerance will have higher AChE concentrations compared to non-induced tadpoles from the same population.

## **4.2 METHODS AND MATERIALS**

### **4.2.1 Insecticide background**

We chose to work with carbaryl (Sevin® 22.5% active ingredient; CAS 63-25-2), an AChE-inhibiting insecticide that currently dominates home insecticide sales and is also applied in agricultural settings for pest and malarial prevention (Grube et al. 2011). The half-life of carbaryl is 10 d at a pH of 7 and the maximum concentration detected aquatic systems is 33.5 ppb (USEPA 2008). Carbaryl can enter amphibian habitats through direct application, drift, and runoff (Mitra et al. 2011) and operates by reversibly binding to AChE. With AChE inhibited, acetylcholine accumulates, leading to overstimulation of neurons and eventually mortality (Brown 2005; Lajmanovich et al. 2010). A well-established physiological mechanism of evolved tolerance in target pest species is the upregulation of AChE (Oakeshott et al. 2005). Thus, a possible mechanism by which amphibians might experience induced tolerance is by upregulating the production of AChE.

### **4.2.2 Experimental design**

To investigate the possibility of induced tolerance in wood frogs, we conducted an embryo-exposure experiment and a hatchling-exposure experiment (Fig. 1). Both experiments consisted of two distinct phases; Phase 1 consisted of an exposure to sublethal concentration of carbaryl to induce tolerance whereas Phase 2 consisted of an exposure to a lethal concentration of carbaryl to quantify time to death (TTD) and assess tolerance. TTD assays are useful tools for assessing

relative tolerance among different groups and are also good indicators of an individual's insecticide tolerance (Semlitsch et al. 2000, Bridges and Semlitsch 2000).

To understand how population proximity to agriculture might impact the ability to induce tolerance, we conducted the embryo and hatchling experiments using individuals from four populations that vary in proximity to agriculture. Past studies have demonstrated that agricultural fields > 200 m from small ponds do not have strong effects on aquatic systems (Declerck et al. 2006), thus we chose two populations from ponds that were close to agricultural fields (< 100 m from the edge of the pond) and two populations that were far from agricultural fields (> 800 m; Table A1). A recent study also demonstrated that tadpoles from these populations also vary in their tolerance to carbaryl (Hua et al. 2013). Tadpoles from populations far from agricultural fields (Hopscotch Pond and Square Pond) are more susceptible to carbaryl than tadpoles from populations close to agricultural fields (Trailer Park Pond and Staub Pond; Table A1). On 13-14 March 2012, we collected seven to ten newly oviposited egg masses from each of the four wood frog populations. We immediately placed all collected egg masses into plastic buckets filled with ~9 L of filtered water. Populations were kept separate but egg clutches from the same population mixed together (pH = 7; Gosner stage 5).

#### **4.2.3 Embryo-exposure experiment**

Phase 1- Sublethal exposure to induce tolerance. Immediately after eggs were collected, we isolated 800 individual embryos from each of the four populations (Gosner stage 5; Gosner 1960) by individually separating an equal number of embryos from each of the egg masses while keeping the jelly coat of each individual embryo intact. After separating the individuals from their egg masses, we distributed individual eggs into a completely randomized, factorial design



with animals from the four populations crossed with four sublethal exposures (nominal concentrations: 0, 0.1, 0.5, or 1 ppm of carbaryl). This produced a total of 16 treatments, which we replicated 4 times each for a total of 64 experimental units. Our experimental units were 500 ml plastic containers filled with 450 ml of well water. From the 800 embryos representing each population, we randomly assigned groups of 200 embryos into each of the four sublethal carbaryl treatments. Each treatment was replicated 4 times (50 individuals/container). We reared the embryos in the laboratory at a constant temperature of 20 °C. Pesticide solutions were not renewed. After being exposed for 60 hrs (Fig. 1), embryos reached pre-hatchling stage (Gosner stage 19). Keeping together individuals in each of the four replicates, tadpoles were transferred to 450 ml of pesticide-free, filtered well water until they hatched. On 17 March, again keeping individuals together by replicate, we transferred all hatchlings to 14-L containers filled with 7 L of pesticide-free well water. Hatchlings were held in clean water for 4 d prior to Phase 2 of the experiment (Gosner 25) and were not fed because they were still living off of yolk reserves.

All eggs not used in the embryo-exposure experiment were placed in 100-L outdoor pools containing 90 L of aged well water (outdoor air temperature ranged from 11°C to 24 °C). We used these eggs in the subsequent hatchling-exposure experiment.

Phase 2- Lethal exposure to assess induced tolerance. Prior to the start of Phase 2, we randomly chose 10 tadpoles from each of the 16 treatments and measured tadpole mass. To determine whether the initial exposure to sublethal concentrations of carbaryl induced tolerance later in life, we crossed the 16 treatments from Phase 1 with two subsequent carbaryl exposures in Phase 2. The two carbaryl treatments in Phase 2 were a no-pesticide control (i.e. water) and a concentration that should be lethal to tadpoles. Using a factorial, completely randomized design, this produced 32 treatments replicated 5 times each, for a total of 160 experimental units. The

experimental units were 100-mL Petri dishes filled with either 70 mL of water or 70 mL of the lethal carbaryl solution.

When conducting TTD assays, the objective is to cause some mortality but not complete and immediate mortality (Newman 2010). For carbaryl, the estimated LC50 value for wood frogs is 1.2 ppm (Relyea 2003) and past studies found that wood frogs were highly susceptible to 30 ppm (Bridges and Semlitsch 2002). Based on these data, we chose an intermediate concentration of 8 ppm of carbaryl for the TTD assay.

On 21 March, we randomly added 10 tadpoles (Gosner stage 25) from each of the 16 embryonic treatments to either water or 8 ppm of carbaryl. To assess tadpole tolerance, we measured TTD and total survival. We assessed tadpole mortality every 4 hrs for 180 hrs and conducted a water change and reapplication of carbaryl every 24 hrs. Due to low mortality (< 1%) after 72 hrs with 8 ppm of carbaryl, we increased the concentration to 15 ppm and continued to observe low mortality (<1%) after 96 hrs. As a result, we further increased the concentration to 18 ppm. These nominal carbaryl concentrations are high relative to what can be found in nature (Norris et al. 1983), but our objective was to discriminate among treatment groups in terms of pesticide tolerance to determine whether prior pesticide exposure led to increased levels of tolerance. We maintained the nominal concentration of 18 ppm for subsequent water changes for 180 hrs (7.5 d). After 180 hrs, we terminated the TTD assay and documented the number of surviving tadpoles. In accordance with standard toxicity tests, tadpoles were not fed during the test (ASTM 2008). The hatchling tadpoles had food reserves in the form of yolk as evidenced by the low mortality (1 to 2% among the four populations; Table A3) observed in animals exposed to 0 ppm of carbaryl in the TTD assay. All methods were approved by the University of Pittsburgh's IACUC (protocol 12050451).

#### **4.2.4 Hatchling-exposure experiment**

To conduct the hatchling-exposure experiment, we followed the same two-phase experimental design as the embryo-exposure experiment using the remaining individuals from each population that we reared in outdoor pools. Because these embryos were reared outside with lower temperatures (but within the range of temperatures experienced in nature), they developed more slowly and this allowed us to stagger the embryo- and hatchling- exposure experiments while using animals from the same genetic backgrounds.

Phase 1- Sublethal exposure to induce tolerance. Using the same method as the embryo-exposure experiment, we took animals from the same four populations and crossed them with four sublethal exposures (nominal concentrations: 0, 0.1, 0.5, or 1 ppm of carbaryl). This produced a total of 16 treatments, which we replicated 4 times each for a total of 64 experimental units. Our experimental units were 14-L plastic containers filled with 7 L of one of the four of sublethal carbaryl solutions. We brought the embryos held outdoors (~550 individuals from each population) back into the lab on 20 March. All embryos hatched indoors (Gosner stage 20) by 21 March and we randomly assigned 128 hatchlings to each of the four sublethal carbaryl treatments (32 individuals/ replicate). On 24 March, after being exposed to these sublethal concentrations of carbaryl for 66 hrs, keeping individuals together within replicates, we transferred all hatchlings (Gosner stage 24) to pesticide-free water for 2 d prior to Phase 2 (Fig. 1).

Phase 2- Lethal exposure to assess induced tolerance. Prior to the TTD assay (Phase 2), we randomly chose 10 tadpoles from each of the 16 treatments and measured tadpole mass. Using the same protocol as the embryonic experiment, we exposed tadpoles (Gosner stage 25) from each of the 16 hatchling treatments to either 0 or 15 ppm of carbaryl. The 32 treatments were replicated 5 times for a total of 160 experimental units (100-mL Petri dishes filled with 70

mL of 0 or 15 ppm of carbaryl). We assessed tadpole mortality every 4 hrs for 92 hrs and changed the Petri dish solutions every 24 hrs. At 92 hrs, we ended the experiment because tadpoles in the 0 ppm treatment of the TTD assay began to experience mortality, although mortality was low (1 to 11% among the four populations; Table A3). At 92 hrs, we documented final tadpole survival.

#### **4.2.5 Pesticide applications**

To generate pesticide solutions, we created a stock solution by dissolving a commercial grade solution of carbaryl (Sevin©) in filtered water. To achieve the stock solutions of the four sublethal concentrations for Phase 1 (0.1, 0.5, and 1 ppm), we added 4, 7, and 15 ul of commercial grade carbaryl with 8.5, 3.5, and 3.5 L of filtered water, respectively. We then added 450 mL of these stock solutions to 500-mL experimental units. For the embryonic TTD assay (Phase 2), we created a stock solution to achieve 8, 15, and 18 ppm by adding 245, 460, and 552 ul of commercial grade carbaryl into 7.2 L of filtered water, respectively. Similarly, for the hatchling TTD assay, to create stock solutions by adding 460 ul to 7.2 L of filtered water to achieve 15 ppm. We then added 70 ml of these stock solutions to each of the Petri dish experimental units. Finally, for both experiments we used filtered water to create the control stock solutions.

#### **4.2.6 Pesticide testing**

To determine the actual concentrations of the pesticides used in this study, we collected samples (500 mL) of each stock solution immediately after animals were added in Phase 1 and after every

water change in Phase 2. Samples were stored in amber jars and kept at 4°C in accordance to established analytical methods (OECD 2007). Samples were sent to an independent laboratory and concentrations were tested using aqueous injection HPLC with post column derivatization (Georgia Chemical Laboratory, Athens, GA, USA). Since we used identical methodologies to generate pesticide solutions for both the embryonic and hatchling exposure experiments, we only sent embryonic exposure experiment samples to be tested.

Stock solutions were analyzed within 5 wks of the sampling date and actual concentrations for the 0.1, 0.5, and 1 ppm nominal concentrations were 0.07, 0.25, and 0.62 ppm of carbaryl. Actual concentrations for the lethal concentrations of 8, 15, and 18 ppm were 3.7, 6.2, and 7.1 ppm. Carbaryl was not detectable in our control treatments (minimum detectability = 0.01 ppm). Despite storing samples in accordance to established analytical methods (OECD 2007), the actual concentrations were lower than nominal concentrations likely due to sample degradation through a variety of biological and chemical processes (Sherma and Beroza 2005). When describing our results, we refer to the actual concentrations.

#### **4.2.7 Acetylcholine esterase assay**

To determine whether any observations of induced tolerance were associated with increases in AChE expression, we measured the concentration of total tadpole AChE in a sample of tadpole bodies. For embryo- and hatchling-exposed tadpoles, we measured AChE concentration prior to Phase 2. In the hatchling-exposure experiment, we also measured the AChE concentration in the surviving tadpoles immediately after Phase 2. Following Phase 2 of the embryo-exposure experiment, several treatments had replicates where no tadpoles survived. Consequently, we

were unable to assess AChE concentrations with equal representation from each replicate thus we did not analyze AChE concentration after Phase 2 for the embryonic-exposure experiment.

To quantify AChE concentrations, we subsampled 10 individuals from each treatment by haphazardly selecting two individuals from each of the five replicates from Phase 2; these 10 individuals were then pooled by treatment. We individually homogenized whole tadpoles with 10% octylphenoxypolyethoxy ethanol (triton X-100) in 0.1 M tris (hydroxymethyl) aminomethane hydrochloride (pH = 7.4) using the Ellman method (1961). The homogenates were centrifuged at 23,447 G for 15 min. AChE concentration was determined using the Ellman colorimetric method (1961). The reaction mixture consisted of 200 ul of tadpole homogenate, 50 ul of 20 mM dithio bis 2-nitrobenzoic acid (DTNB) and 50 ul of 20 mM acetylthiocholine (AcSCh) in 96-well plates. After a 15-min incubation period at 20°C, each sample was assayed in duplicate by measuring absorbance at 405 nm using an Epoch 96-well plate spectrophotometer.

#### **4.2.8 Statistical analysis**

To investigate the possibility of induced tolerance, we compared rates of tadpole survival in the TTD assay when previously exposed to different sublethal insecticide concentrations. We analyzed the data from the embryonic and hatchling experiment using a separate Cox's proportional hazards model for each population (Cox 1972). Using this method of survival analysis, we used the TTD of each individual tadpole to determine hazard ratios, which examine the probability of mortality of animals previously exposed to various sublethal carbaryl concentrations in Phase 1 relative to animals exposed to 0 ppm in Phase 1. A hazard ratio <1 for tadpoles that were initially exposed to insecticides as embryos or hatchlings indicates a decrease

in the probability of mortality compared to individuals exposed to no insecticides as embryos or hatchlings. In contrast, a hazard ratio  $<0$  for tadpoles that were initially exposed to insecticides as embryos or hatchlings indicates an increase in the probability of mortality compared to individuals exposed to no insecticides as embryos or hatchlings. We define induced tolerance as cases where treatment hazard ratios are significantly lower than the control (hazard ratio = 0).

Following the time to death assay, we also analyzed survival of tadpoles in each experimental unit (Petri dish) using an ANOVA. Significant univariate results were assessed using Tukey's pairwise comparison test and significant interactions were analyzed using targeted post-hoc tests that separately compared the effect of each sublethal concentration within a population. Although less sensitive, the survival results were consistent with TTD results. Thus, we do not present and discuss the ANOVA results for survival in the main text (but see Digital Appendix, Figs. A1 and A2, and Table A4).

For the embryo-exposure experiment, we analyzed the AChE concentration of tadpoles prior to the TTD assay using an ANOVA. Significant univariate results were assessed using Tukey's pairwise comparison test and significant interactions were analyzed using targeted post-hoc tests that separately compared the effect of each sublethal concentration for each population. Because we subsampled 10 tadpoles from five replicates and did not track which tadpoles came from each replicate, we could not take the mean AChE from each replicate. Instead, we had to analyze the data by taking the mean of the 10 samples from each treatment. To guard against inflated degrees of freedom associated with this pseudoreplication, we conducted the analysis using the degrees of freedom based on the number of independent replicate containers (i.e. five) rather than on the number of tadpoles actually measured (i.e. 10).

For the hatchling-exposure experiment, we analyzed the AChE concentration of tadpoles before and after Phase 2 using separate ANOVAs. We did not use a repeated-measures analysis because different individuals were measured before and after Phase 2. We conducted these analyses in the same manner as the embryo-exposure experiment.

Finally, we used an ANOVA to confirm there were no differences in mass by comparing mass of tadpoles prior to Phase 2 in both the embryo- and hatchling-exposure experiments.

### **4.3 RESULTS**

#### **4.3.1 Time to death in Phase 2: Embryo-exposure experiment**

To investigate the possibility of induced tolerance, we compared rates of tadpole survival in the TTD assay when previously exposed to different sublethal insecticide concentrations. We analyzed the data from the embryonic and hatchling experiment using a separate Cox's proportional hazards model for each population (Cox 1972). Using this method of survival analysis, we used the TTD of each individual tadpole to determine hazard ratios, which examine the probability of mortality of animals previously exposed to various sublethal carbaryl concentrations in Phase 1 relative to animals exposed to 0 ppm in Phase 1. A hazard ratio  $<1$  for tadpoles that were initially exposed to insecticides as embryos or hatchlings indicates a decrease in the probability of mortality compared to individuals exposed to no insecticides as embryos or hatchlings. In contrast, a hazard ratio  $>1$  for tadpoles that were initially exposed to insecticides as embryos or hatchlings indicates an increase in the probability of mortality compared to



individuals exposed to no insecticides as embryos or hatchlings. We define induced tolerance as cases where treatment hazard ratios are significantly lower than the control (hazard ratio = 0).

Following the time to death assay, we also analyzed survival of tadpoles in each experimental unit (Petri dish) using an ANOVA. Significant univariate results were assessed using Tukey's pairwise comparison test and significant interactions were analyzed using targeted post-hoc tests that separately compared the effect of each sublethal concentration within a population. Although less sensitive, the survival results were consistent with TTD results. Thus, we do not present and discuss the ANOVA results for survival in the main text (but see Digital Appendix, Figs. A1 and A2, and Table A4).

For the embryo-exposure experiment, we analyzed the AChE concentration of tadpoles prior to the TTD assay using an ANOVA. Significant univariate results were assessed using Tukey's pairwise comparison test and significant interactions were analyzed using targeted post-hoc tests that separately compared the effect of each sublethal concentration for each population. Because we subsampled 10 tadpoles from five replicates and did not track which tadpoles came from each replicate, we could not take the mean AChE from each replicate. Instead, we had to analyze the data by taking the mean of the 10 samples from each treatment. To guard against inflated degrees of freedom associated with this pseudoreplication, we conducted the analysis using the degrees of freedom based on the number of independent replicate containers (i.e. five) rather than on the number of tadpoles actually measured (i.e. 10).

For the hatchling-exposure experiment, we analyzed the AChE concentration of tadpoles before and after Phase 2 using separate ANOVAs. We did not use a repeated-measures analysis because different individuals were measured before and after Phase 2. We conducted these analyses in the same manner as the embryo-exposure experiment.

Finally, we used an ANOVA to confirm there were no differences in mass by comparing mass of tadpoles prior to Phase 2 in both the embryo- and hatchling-exposure experiments.

#### **4.3.2 Time to death in Phase 2: Hatchling- exposure experiment**

Hatchling exposure to sublethal carbaryl concentrations during Phase 1 did not affect tadpole mass prior to Phase 2 ( $F_{3,144} = 0.88$ ;  $p = 0.45$ ). We also found low mortality in the control treatment of the TTD assay (Average  $\pm$  SE; Hopscotch =  $73\% \pm 4.5$ ; Square =  $90\% \pm 3.1$ ; Staub =  $92\% \pm 3.5$ ; Trailer =  $96\% \pm 1.9$ ). When we conducted the Cox proportional hazard test, we found that the initial exposure of hatchlings to sublethal carbaryl had no subsequent effect on the mortality of tadpoles from populations located close to agriculture (Staub and Trailer Park Ponds) when exposed to lethal carbaryl concentrations (Table 2; Figs 3a, b). In contrast, tadpoles from the populations located far from agriculture (Hopscotch and Square Ponds) exhibited increased tolerance when exposed to lethal carbaryl concentrations. For Hopscotch Pond, tadpoles that were initially exposed to 0.07, 0.25 and 0.62 ppm of carbaryl as hatchlings became more tolerant to the subsequent lethal carbaryl concentration than tadpoles that were not initially exposed (Table 2; Figs 3c, d). For Square Pond, only tadpoles exposed to 0.62 ppm as hatchlings were more tolerant to a lethal concentration of carbaryl than tadpoles that were not exposed to carbaryl as hatchlings.

#### **4.3.3 Acetylcholine esterase concentrations: Embryo-exposure experiment**

For the embryo-exposure experiment, we assessed AChE concentrations of all four populations before Phase 2 and found significant effects of population, concentration, and their interaction

(Table 3A). Post-hoc comparisons indicate a significant effect of sublethal pesticide exposure on tadpoles from Hopscotch (far), Square (far), and Trailer Park Pond (close), but not on Staub Pond (close; Table 3B). Additionally, tadpoles from Trailer Park pond that were initially exposed to intermediate carbaryl concentrations (0.07 and 0.25 ppm) had significantly higher AChE concentrations than the control (Fig. 4). Tadpoles from Hopscotch Pond exposed to 0.07 and 0.25 ppm of carbaryl had significantly lower AChE concentrations than the control (Fig. 4). Tadpoles from Square Pond that were initially exposed to the highest concentration of carbaryl (0.62 ppm) had higher AChE concentrations than the control.

#### **4.3.4 Acetylcholine esterase concentrations: Hatchling-exposure experiment**

For the hatchling-exposure experiment, we assessed AChE concentrations on tadpoles that had been initially exposed to sublethal carbaryl concentrations before Phase 2 and again on tadpoles that survived Phase 2. In the first analysis, we found an effect of population and a population-by-concentration interaction (Table 3A). Our post-hoc comparisons found no significant effects of sublethal carbaryl exposure concentrations within each of the populations (Table 3B; Fig. 5).

In the analysis of tadpoles that survived Phase 2, we found a significant effect of population and concentration but no interaction (Table 3A). Our post-hoc comparisons found no differences among the four populations. In contrast, of the tadpoles that survived the TTD assay, those that were exposed to sublethal concentrations of carbaryl as hatchlings had significantly higher AChE concentrations compared to tadpoles not exposed to carbaryl as hatchlings.

#### 4.4 DISCUSSION

We discovered that exposing amphibian embryos and hatchlings to sublethal concentrations of carbaryl can induce increased tolerance later in life. Interestingly, the pattern of induced tolerance only emerged in tadpoles from populations that were far from agricultural fields. For both far from agriculture populations, insecticide concentration was important to predicting patterns of induced tolerance. Generally, for both the embryo and hatchling experiments, as the concentration of the initial sublethal application increased, the magnitude of induced tolerance also increased. However, this was not the case for Square Pond (far); tadpoles exposed to the highest sublethal concentration (0.62 ppm) as embryos became less tolerant to a subsequent lethal exposure of carbaryl. In our assessment of AChE, we found that exposure to sublethal concentrations both significantly increased and decreased tadpole AChE concentrations.

Plasticity theory predicts that populations that experience variable environmental conditions should maintain the ability to induce adaptive phenotypes in response to changing environments (Schlichting and Pigliucci 1998). In contrast, less variable environments should select for constitutive expression of traits and decreased plasticity. Consistent with this theory, we only observed induced tolerance in populations that were far from agriculture and would be unlikely to consistently experience insecticides (ponds < 200 m from agriculture are not strongly affected by insecticides; Declerck et al. 2006). These populations might occasionally experience insecticides, such as those sprayed in forests to control tree-damaging insects, but such applications would not be as consistent over the years as insecticide applications on agricultural crops. Considerable effort has sought to understand insecticide resistance that arises via constitutive genetic pathways (i.e. evolved resistance; Lopes et al. 2008), but we demonstrate that for populations that experience less consistent insecticide exposures, induced tolerance may

be an important alternative mechanism in which evolved resistance can be achieved, which suggests the process of genetic assimilation. An important next step is to determine the generalizability of this pattern across a broader agricultural gradient. Further, despite higher constitutive tolerances of populations close to agriculture, the lack of a plastic response suggest that there may be costs to maintaining constitutive resistance (Callahan et al. 2008). Future studies investigating these potential costs are critical to understanding the relative contribution of evolved resistance versus induced tolerance in natural systems exposed to insecticides.

To our knowledge, this is the first study in amphibians and only the second study among all animals to demonstrate that sublethal and ecologically relevant concentrations of a common insecticide can induce increased tolerance. The only other study was that of Poupardin et al. (2008), who exposed mosquito larvae for 24 hrs to sublethal concentrations of two insecticides (permethrin and temephos), and found that pre-exposed larvae subsequently had increased tolerance. Poupardin et al. (2012) went on to show that the insecticides select for different genes in individuals previously exposed to insecticides compared to those not previously exposed. Thus, the authors suggest that the ability to detect and induce responses to insecticides can have long-term impacts on how insecticides select for resistance. Future studies should investigate the contribution of induced tolerance to shaping the evolution of resistance.

We also found that the concentration of the initial pesticide exposure affected the patterns of induced tolerance. Generally, only the intermediate (0.25 ppm) and highest (0.62 ppm) sublethal concentrations resulted in induced tolerance. For both embryonic and hatchling experiments, there was only one case (tadpoles from Hopscotch [far] that were exposed as hatchlings) where exposure to the lowest sublethal concentration of carbaryl (0.07 ppm) led to induced tolerance. Thus, similar to threshold limits necessary for plastic responses of prey to

predator cues (Relyea 2004; McCoy et al. 2012), a threshold insecticide concentration must also be met in order for the induction of tolerance. In nature, aquatic systems are often commonly exposed to low concentrations of insecticides ( $<1$  ppm). Though low concentrations of chemicals are often not lethal to many taxa, the induction of tolerance following exposure to these low concentrations could potentially play a significant role in understanding population persistence following insecticide contamination.

Though we generally found that sublethal concentrations of insecticides induced increased tolerance, exposure of embryos from Square Pond (far) to the highest sublethal concentration of carbaryl induced a decrease in tolerance. For this population, we found that sublethal concentrations initiated a nonlinear hormetic dose response. A hormetic dose response is commonly found in response to environmental toxins (Costantini et al. 2010) and occurs when exposure to a high concentration of a chemical agent or environmental factor is damaging to an individual but lower exposures are beneficial to the individual compared to a control (Mattson 2008, Hayes et al. 2002). The results for Square Pond (far) indicate that the inducibility of tolerance for certain populations may be confined to a narrow range of concentrations. To understand the contribution of induced tolerance in natural systems, an important challenge is to identify the concentration windows in which induced tolerance is increased versus decreased.

Ontogenetic variation in insecticide tolerance is a broadly documented pattern and we expected the ability to induce tolerance would vary across ontogeny. However, we found that embryos and hatchlings were both capable of experiencing induced tolerance. The ability for individuals to induce tolerance in early life stages may have significant lasting consequences in later life stages by decreasing the lag time between the exposure to a contaminant and the ability to mount adaptive responses (Ferrari and Chivers 2009). The majority of toxicological studies

focus on assessing tolerance at single life stages and few studies have considered how insecticide exposure early in development may increase future susceptibility (Bridges 2000; Jones et al. 2010). Amphibians are currently experiencing worldwide population declines for a variety of hypothesized reasons including exposure to insecticides (Sparling and Fellers 2007). The ability to induce tolerance to insecticides during more tolerant stages of ontogeny may have significant conservation implications for amphibians exposed to insecticides during more susceptible life stages.

In our assessment of AChE concentrations, we found that exposure to sublethal concentrations of carbaryl early in development can increase AChE concentrations in tadpoles. This pattern was especially prevalent for tadpoles that survived the TTD assay. Indeed, for the 12 treatments where tadpoles were exposed to sublethal concentrations of carbaryl as hatchlings, we found a significant increase in AChE concentration compared to tadpoles not exposed to sublethal carbaryl. A well-established physiological mechanism of tolerance in pest species is the upregulation of AChE (Oakeshott et al. 2005). Thus, a possible mechanism in which amphibians might experience induced tolerance is by upregulating AChE. Consistent with our hypothesis, the most tolerant individuals (survivors of TTD assay) from populations far from agriculture (Square and Hopscotch) in the hatchling experiment had significantly more AChE compared to the animals from the control treatment.

In contrast, for both embryo and hatchling experiments, when we measured AChE concentrations of tadpoles prior to the TTD assay, changes in AChE concentrations were less prevalent. Of the 24 total cases where embryos or hatchlings were exposed to sublethal carbaryl, we only found five cases (all in the embryo-exposed experiment) where sublethal exposure to carbaryl caused a significant change in AChE concentrations relative to the control. These data

suggest that the pattern of increased AChE may be detectable only in the most tolerant individuals. In addition to being less prevalent, of the five cases where AChE concentrations were significantly affected, we found that the patterns of AChE concentration were not consistent with our predictions of induced tolerance. For three of these cases, sublethal concentrations of carbaryl caused an increase in AChE concentrations but there was no evidence of induced tolerance. For the other two cases, sublethal concentrations of carbaryl caused decreased AChE concentrations. Though we show that early exposure to sublethal concentrations of carbaryl can induce changes in AChE concentrations at later life stages, it is also possible that AChE may not be the only mechanism that allows for induced tolerance. Future studies are critical to identifying the relative contribution of AChE to induced tolerance.

### Conclusions

In rapidly changing environmental conditions, the contribution of plasticity has critical implications for individuals and the evolution of populations by allowing adaptive traits to be induced rapidly within a single generation. We are the first to demonstrate that sublethal and ecologically relevant concentrations of a common insecticide can, within the same generation, induce adaptive tolerance in amphibians. Understanding the role of induced tolerance can have significant conservation implications for populations of non-target species exposed insecticide contaminants. Specifically, the pattern of inducibility varies by population but appears to be consistent with plasticity theory. Patterns of induced tolerance are dependent on insecticide concentration and occur at very early life stages. Finally, in our analysis of AChE concentrations, we find that exposure to sublethal concentrations has a lasting legacy on tadpole AChE concentrations. To sum, inducible responses to anthropogenic disturbances may have a significant impact on shaping patterns of species abundance. Future work identifying the



underlying mechanisms that drive these inducible responses to insecticides as well as other anthropogenic contaminants are an important step towards understanding the long-term impact of disturbances on natural systems.

**Table 4-1. Hazard ratios for tadpoles from four populations that had been previously exposed to four sublethal concentrations of carbaryl as embryos (0, 0.1, 0.5 or 1.0 ppm) and then re-exposed as tadpoles to a lethal concentration of carbaryl (18 ppm). Negative hazard ratios indicate that the initial exposure made tadpole more tolerant whereas positive hazard ratios indicate that the initial exposure made tadpole less tolerant.**

<b>Population</b>	<b>Hazard ratios for the initial carbaryl exposures as embryos (p-value)</b>			<b>Percent censored</b>
	0.07 ppm	0.25 ppm	0.62 ppm	
Hopscotch	-0.28 (0.21)	-0.82 ( <b>&lt;0.001</b> )	-0.54 ( <b>0.019</b> )	25%
Square	-0.4 (0.22)	-1.2 ( <b>0.003</b> )	1.07 ( <b>&lt;0.001</b> )	57%
Staub	-0.27 (0.25)	-0.31 (0.18)	0.06 (0.77)	23%
Trailer Park	-0.33 (0.16)	-0.32 (0.17)	-0.24 (0.30)	24%

**Table 4-2. Hazard ratios for tadpoles from four populations that had been previously exposed to four sublethal concentrations of carbaryl as hatchlings (0, 0.1, 0.5 or 1.0 ppm) and then re-exposed as tadpoles to a lethal concentration of carbaryl (15 ppm). Negative hazard ratios indicate that the initial exposure made tadpole more tolerant whereas positive hazard ratios indicate that the initial exposure made tadpole less tolerant.**

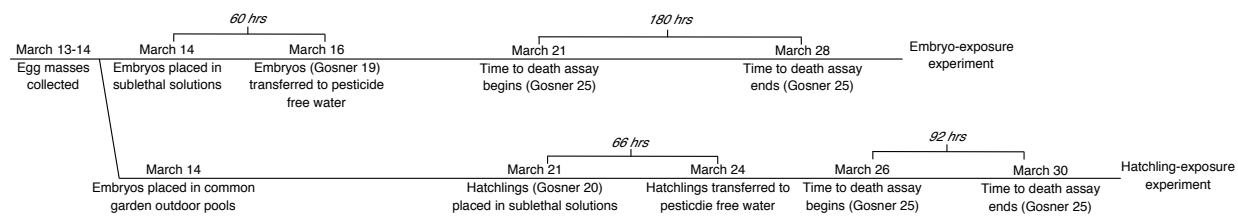
<b>Population</b>	<b>Hazard ratios for the initial carbaryl exposures as hatchlings (p-value)</b>			<b>Percent censored</b>
	0.07 ppm	0.25 ppm	0.62 ppm	
Hopscotch	-0.64 <b>(0.023)</b>	-0.65 <b>(0.024)</b>	-0.83 <b>(0.004)</b>	55%
Square	-0.62 (0.06)	-0.45 (0.17)	-0.91 <b>(0.013)</b>	68%
Staub	-0.47 (0.27)	0.18 (0.63)	0.34 (0.34)	71%
Trailer Park	0.1 (0.77)	-0.53 (0.21)	-0.37 (0.36)	74.5%

**Table 4-3. A) Test results from ANOVAs on AChE concentrations in tadpoles 1) before Phase 2 in the embryo-exposure experiment, 2) before Phase 2 in the hatchling exposure, and 3) after Phase 2 in the embryo exposure experiment. B) AChE concentrations ANOVA by population from tadpoles: 1) before Phase 2 in the embryo-exposure experiment and 2) before Phase 2 in the hatchling-exposure experiment**

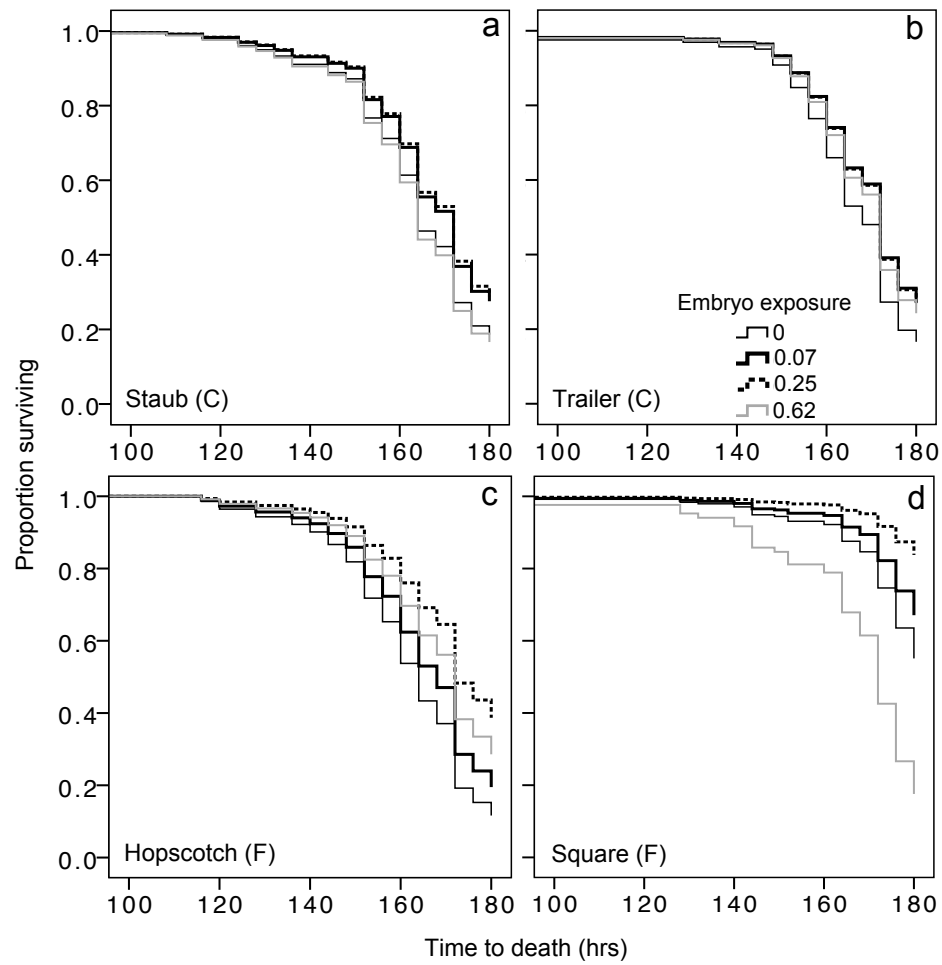
A. AChE concentration ANOVA results		df	F	P-value
1. Embryo exposure, before Phase 2	Population	3, 48	14.7	<b>&lt;0.001</b>
	Concentration	3, 48	7.0	<b>&lt;0.001</b>
	Pop'n x Conc	9, 48	10.8	<b>&lt;0.001</b>
2. Hatchling exposure, before Phase 2	Population	3, 48	5.62	<b>0.003</b>
	Concentration	3, 48	0.57	0.64
	Pop'n x Conc	9, 48	2.101	<b>0.047</b>
3. Hatchling exposure, after Phase 2	Population	3, 64	3.1	<b>0.03</b>
	Concentration	3, 64	9.7	<b>&lt;0.001</b>
	Pop'n x Conc	9, 64	0.98	0.38

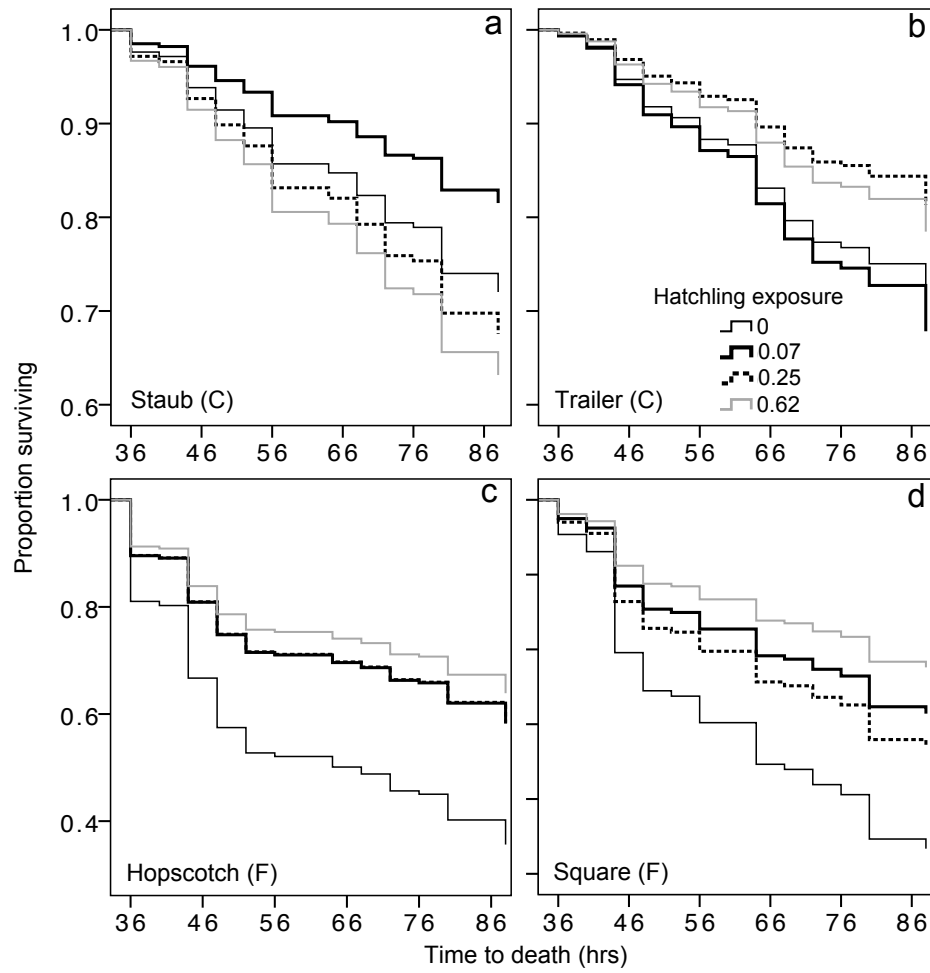
1. Embryo exposure, before Phase 2	Hopscotch pond (df = 3, 12)	Square pond (df = 3, 12)	Trailer pond (df = 3, 12)	Staub pond (df = 3, 12)
Pesticide	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.17
2. Hatchling exposure, before Phase 2	Hopscotch pond (df = 3, 12)	Square pond (df = 3, 12)	Trailer pond (df = 3, 12)	Staub pond (df = 3, 12)
Pesticide	0.18	0.20	0.06	0.43



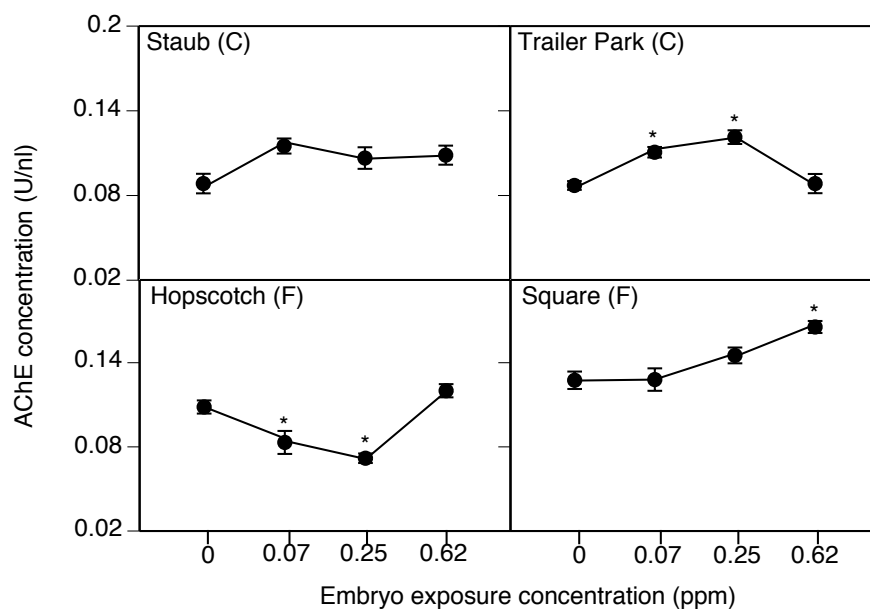
**Figure 4-1. Experiment timeline.**



**Figure 4-2. Survival across time of tadpoles exposed to lethal carbaryl as embryos. The abbreviation “F” indicates populations far from agricultural fields (>800 m) whereas “C” indicates populations close to agricultural fields (<100 m).**

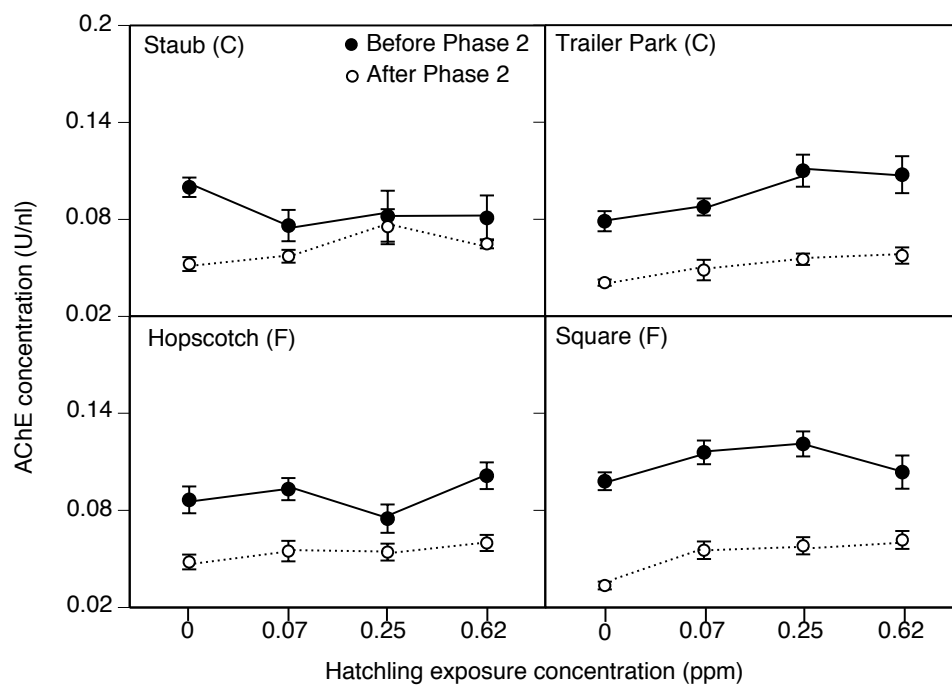


**Figure 4-3. Survival across time of tadpoles exposed to lethal carbaryl as hatchlings. The abbreviation “F” indicates populations far from agricultural fields (>800 m) whereas “C” indicates populations close to agricultural fields (<100 m).**



**Figure 4-4. Acetylcholine esterase concentrations of tadpoles exposed to sublethal concentrations of carbaryl as embryos before Phase 2. Asterisks represent significant differences ( $p < 0.05$ ) in tadpole AChE concentration between hatchlings that were not initially exposed to carbaryl as embryos and hatchlings that were initially exposed to carbaryl.**





**Figure 4-5. Acetylcholine esterase concentration of tadpoles exposed to sublethal concentrations of carbaryl before and after Phase 2. Closed circles represent AChE concentrations of tadpoles measured before Phase 2 and opened circles represent AChE concentrations measured after Phase 2.**

## **5.0 INDUCED TOLERANCE FROM A SUBLETHAL INSECTICIDE LEADS TO CROSS-TOLERANCE TO OTHER INSECTICIDES**

### **5.1 INTRODUCTION**

Pesticides are important tools for disease prevention and agricultural production. However, as the use of pesticides continues to grow, understanding their effects on natural systems will become increasingly important (Laurance 2001). An issue that has received substantial attention is the evolution of insecticide tolerance in targeted pest species (Georghiou 1990). While insecticide tolerance in pest or vector species causes over \$1.5 billion of losses each year, (Pimentel 2005) the same phenomenon may positively affect non-target species by facilitating population persistence following insecticide contamination (Jansen et al. 2011a). Interestingly, despite the conservation implications of increased insecticide tolerance, our understanding of tolerance in non-target organisms is extremely limited.

Insecticide tolerance is almost exclusively described as a constitutive trait that arises from the microevolution of decreased susceptibility over time (Feyereisen 1995a; Pimentel 2005; Lopes et al. 2008). However, another process that allows for increased tolerance is the induction of tolerance through phenotypic plasticity, which is defined as the capacity of a single genotype to exhibit variable phenotypes in different environments (Schlichting and Pigliucci 1998). In this scenario, exposure to sublethal insecticide concentrations can induce increased tolerance to a

lethal concentration of the same pesticide later in life. Past studies have demonstrated that exposure to other contaminants (i.e. heavy metals) early in development can lead to positive effects later in life, but this phenomenon has rarely been considered in the context of pesticides (Costantini et al. 2012). Currently this phenomenon is only known to occur in one species of invertebrate (i.e. a mosquito [*Aedes aegypti*]; Poupardin et al. 2008b; Poupardin et al. 2012) and one species of vertebrate (wood frogs [*Lithobates sylvaticus*]; Hua et al. 2013b). It is possible that many other species have this ability, but little investigation has been conducted, likely because induced tolerance does not fit the current paradigm of constitutive pesticide tolerance. In the few studies that have been conducted, researchers have found that the concentration of initial sublethal exposure and the proximity of the population to agricultural fields (i.e. a proxy for the frequency of pesticide exposure) can affect the existence of induced tolerance (Hua et al. 2013b).

Given the enormity of available pesticides (over 1055 registered active ingredients; USEPA 2007) induced pesticide tolerance would be particularly beneficial to non-target species if it were to confer increased tolerance not only against the pesticide it first experienced, but also against many other pesticides (e.g., induced cross-tolerance). Constitutive cross-tolerance is frequently observed in targeted pest species (Tabashnik 1989; Rodríguez et al. 2002) and has been documented in a few non-target species (Brausch and Smith 2009; Hua et al. 2013a). It is most common among pesticides with similar modes of action, although constitutive cross-tolerance can also occur among pesticides with different modes of action (Liu and Yue 2000; Alou et al. 2010). This raises the possibility that induced tolerance might also result in induced cross-tolerance to other pesticides. Such cross-tolerance would have major conservation implications for populations exposed to multiple pesticides. We are not aware of any study that has tested for pesticide-induced cross-tolerance in any species.

Amphibians are excellent model organisms to study the possibility of induced cross-tolerance. Amphibians are able to respond plastically to various stressors in their environment (i.e. competitors, predators, pesticides) by altering behavioral and morphological traits (Relyea 2001; Van Buskirk 2009; Relyea 2012). Particular to pesticides, wood frog populations living far from agricultural fields respond to sublethal concentrations of the insecticide carbaryl by inducing increased tolerance to lethal concentrations of carbaryl later in life. Indeed, wood frogs can be induced both as embryos and as newly hatched tadpoles. Further, carbaryl is an inhibitor of acetylcholine esterase (AChE), and induced wood frogs that survive a high dose of carbaryl (18 mg/L) have increased AChE concentrations (Hua et al. 2013b). In contrast, wood frog populations living close to agricultural fields do not exhibit induced tolerance.

Amphibian populations likely encounter a number of insecticides that differ in mode of action (De Meester et al. 2005; Gilliom 2007) and past studies have found that non-induced wood frog populations (i.e. animals that did not receive an initial sublethal exposure to carbaryl) exhibit cross-tolerance to different insecticides that share the same mode of action (Hua et al. 2013a). Thus, it is possible that amphibians induced by sublethal concentrations of one insecticide might induce increased tolerance to many other insecticides.

Using larval wood frogs, the goal of our study was to determine whether an embryonic exposure to sublethal concentrations of carbaryl, which induces higher tolerance to lethal concentrations of carbaryl later in life, could also induce higher tolerance to other insecticides that possess either the same or different modes of actions. Since the upregulation of AChE is a likely mechanism by which amphibians might induce increased tolerance to AChE inhibiting insecticides (Hua et al. 2013b), we hypothesized that embryonic exposure to sublethal concentrations of an insecticide would induce higher tolerance to a later exposure of insecticides

with the same mode of action but would not induce tolerance to a later exposure of insecticides with a different mode of action.

## **5.2 METHODS AND MATERIALS**

### **5.2.1 Insecticide background**

To induce tolerance, we used carbaryl, an insecticide that dominates home insecticide sales and is commonly applied in agricultural settings for pest control and disease prevention (Grube et al. 2011). The half-life of carbaryl is 10 d at a pH of 7 and the range of concentration reported in aquatic systems is 0.73- 1.5 mg/L (Table 1; USEPA 2008). Carbaryl operates by reversibly binding to AChE. Inhibition of AChE causes acetylcholine to accumulate, leading to overstimulation of neurons and eventually mortality (Brown 2005; Lajmanovich et al. 2010a).

To investigate the possibility of induced cross-tolerance, we chose four other commonly applied insecticides: chlorpyrifos, malathion, cypermethrin, and permethrin (Kreidich et al. 2005; Gilliom 2007). Chlorpyrifos and malathion are organophosphates and they have the same mode of action as carbaryl (i.e. an inhibitor of AChE). However, while carbaryl binds reversibly, chlorpyrifos and malathion both bind irreversibly to AChE (Main 1969). Cypermethrin and permethrin are pyrethroids; their mode of action is to interfere with sodium channel function (Newman 2010). Thus, the mode of action of the two pyrethroids differs from carbaryl.

### **5.2.2 Experimental design**

Using a two-phase experiment<sup>2</sup> similar to that of Hua et al (2013b) we tested for induced cross-tolerance in wood frogs collected from two populations that have been previously shown to exhibit induced tolerance when exposed to sublethal concentrations of carbaryl (Hopscotch Pond and Square Pond; Hua et al. 2013b). In Phase 1 of the experiment, we exposed wood frogs to a control and two sublethal carbaryl treatments to induce tolerance. In Phase 2 of the experiment, we tested whether the sublethal exposures to carbaryl induced an increase in tolerance to carbaryl and the other four insecticides later in life. We assessed tolerance by using a time to death (TTD) assay and survival analysis, which is commonly used for assessing relative tolerance among different experimental groups (Bridges and Semlitsch 2000; Semlitsch et al. 2000b; Cothran et al. 2013).

Due to differences in oviposition timing, we collected 15 and 7 newly oviposited egg masses from Hopscotch and Square Ponds on 2 and 8 April 2013, respectively. For both populations, we immediately placed the egg masses into plastic buckets filled with ~9 L of carbon-filtered, UV-treated water (Gosner stage 3).<sup>31</sup> To control for the differences due to oviposition timing between the two populations, we conducted separate experiments for each population.

### **5.2.3 Phase 1- Inducing higher tolerance**

For both populations, within 2 hrs of collection, we isolated 1,200 individual embryos (Gosner stage 4) by individually separating an equal number of embryos from each of the egg masses. In doing so, we took care to keep the jelly coat of each embryo intact. We then distributed

individual eggs into a control or one of two sublethal carbaryl exposures (nominal concentrations: 0.5 or 1 mg/L of carbaryl; Sevin© 22.5% active ingredient; CAS 63-25-2). We chose these concentrations because past studies indicate they induce tolerance without causing mortality (Hua et al. 2013b). We replicated each exposure five times each for a total of 15 experimental units. Our experimental units were 500-ml plastic containers filled with 450 ml of well water and 80 eggs per container. We reared the embryos in the laboratory at a constant temperature of 20 °C on a 16:8 light dark cycle and the insecticide solutions were not renewed. Once all individuals reached Gosner stage 19 (Hopscotch Pond: 6 April; Square Pond: 11 April), we transferred the hatchlings to 14-L containers filled with 7-L of insecticide-free, UV-irradiated, carbon-filtered well water and made sure that we kept all individuals from each experimental unit together. The hatchlings were held in clean water until all individuals reached Gosner stage 25. Hatchlings were not fed because they were still living on their yolk reserves.

#### **5.2.4 Phase 2- Lethal exposure to assess induced tolerance.**

Hopscotch Pond— Once tadpoles from Hopscotch Pond reached Gosner 25 (10 April), we crossed the three sublethal treatments from Phase 1 with a control and five lethal insecticide treatments in a TTD assay. When conducting TTD assays, the objective is to cause some mortality but not complete and immediate mortality (Newman 2010). Thus, to discriminate whether prior carbaryl exposure led to increased tolerance, we chose different lethal concentrations for each insecticide based on past studies (Hua et al. 2013b) and our own pilot data: 0 mg/L control, 15 mg/L of carbaryl (Sevin© 22.5% active ingredient), 5 mg/L of chlorpyrifos (technical grade; CAS 523-15-07-8), 15 mg/L of malathion (technical grade; CAS 121-75-5), 0.03 mg/L of cypermethrin (Hot Shot © 26% a.i.; CAS 523-15-07-8), and 0.1 mg/L of permethrin (technical grade; CAS

526-45-53-1). Using a factorial, completely randomized design, this produced 18 treatments replicated five times each, for a total of 90 experimental units.

The experimental units were 100-mL, glass Petri dishes filled with either 70 mL of water (control) or 70 mL of the lethal insecticide solution. Keeping individuals from Phase I replicates together, we haphazardly assigned 10 tadpoles to either the no-insecticide control or a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin. We conducted water changes every 24 hrs with a renewal of the pesticide concentrations at each water change. To assess tadpole tolerance using TTD, we monitored tadpole mortality every 4 hrs and terminated the experiment after 120 hrs. In accordance with standard toxicity tests, tadpoles were not fed during the test (ASTM 2008). The hatchling tadpoles had food reserves in the form of yolk as evidenced by the low mortality observed in animals exposed to the no-insecticide control in the TTD assay (Hopscotch Pond = 0%; Square Pond = 2%). All methods were approved by the University of Pittsburgh's IACUC (protocol 12050451).

Square Pond– Once tadpoles from Square Pond reached Gosner stage 25 (16 April), we conducted a TTD assay using similar methodology described for tadpoles from Hopscotch Pond. However, we made two modifications. First, due to faster mortality rates of tadpoles from Square Pond, we terminated the experiment after 96 hrs rather than 120 hrs. Second, because 0.1 mg/L of permethrin did not cause sufficient mortality in the TTD assay for Hopscotch Pond (< 7% mortality), we increased the concentration of permethrin from 0.1 to 0.5 mg/L.

### **5.2.5 Insecticide applications**

For Phase 1 (induction of tolerance), we created a working solution by dissolving a commercial grade solution of carbaryl (22.5% Sevin©) in filtered water (pH = 7). To achieve 0.5 and 1 mg/L



of carbaryl, we added 7.5 and 15 ul of commercial grade carbaryl to 3.5 L of filtered water, respectively. We added 450 ml of the carbaryl solution to each of the 500-ml experimental units.

For Phase 2, we first dissolved technical-grade insecticides (malathion, chlorpyrifos, and permethrin) into an ETOH vehicle (Table S1) to create stock solutions. We did not include an ETOH vehicle control in this study since past studies have demonstrated that solvent concentrations higher than we used do not affect tadpole mortality.(Jones et al. 2009) To prepare the working solutions of each insecticide, we added the concentrated stock solutions of malathion, chlorpyrifos, and permethrin or the formulated product of carbaryl and cypermethrin to filtered water (Table S1). We then added 70 ml of these working solutions to each of the Petri dishes. After adding the insecticide solutions, we added ten tadpoles to each Petri dish. Finally, we used filtered water to create the control solutions.

#### **5.2.6 Insecticide testing**

To determine the actual concentrations of insecticides used in this study, we collected 500-mL samples of each working solution after embryos were added in Phase 1 and after tadpoles were added into Petri dishes at Phase 2. Despite storing samples in accordance to established analytical methods (OECD 2007), the samples from Phase 2 experienced degradation due to an extended storage period of 8 wks. Given the discrepancy between actual and nominal concentrations, we conducted an additional experiment to confirm that the difference in concentration was due to sample degradation and not a flaw in our experimental protocol (see Supporting Information for complete details regarding insecticide sampling, chemical analyses, and actual insecticide concentrations). The original and additional chemical analyses, combined with our conversation with the analytical lab personnel, indicate that our experimental protocols

were appropriate for obtaining the nominal concentrations and the discrepancy between actual and nominal concentration was due to degradation of our sample (Perkins 2014). To avoid confusion, when describing our results, we will refer to 0, 0.5, or 1.0 mg/L carbaryl when describing the induction treatments (i.e. Phase 1) because the actual concentrations were close to the nominal concentrations. For our lethal exposures (i.e. Phase 2), the actual concentrations tested were not always close to the nominal treatments, so we will refer to these treatments simply as lethal carbaryl, lethal chlorpyrifos, lethal malathion, lethal cypermethrin, or lethal permethrin.

### **5.2.7 Statistical analysis**

To test for the presence of induced tolerance and induced cross-tolerance, we compared rates of tadpole survival in the TTD assay when previously exposed to three sublethal carbaryl concentrations. We separately analyzed the data for each lethal insecticide treatment using Cox's proportional hazards model for each population (SPSS; Cox 1972) Using this method of survival analysis, we then used the TTD of individual tadpoles to determine hazard ratios, which examine the probability of mortality in animals previously exposed to sublethal carbaryl concentrations (0.5 and 1 mg/L) and in Phase 1 compared to animals previously exposed to 0 mg/L of carbaryl in Phase 1 (Hua et al. 2013b). A hazard ratio  $< 0$  indicates a decrease in the probability of mortality if the animals were previously exposed to sublethal carbaryl whereas a hazard ratio  $> 0$  indicates the reverse outcome. Finally, we also used the Cox regression analysis to compare the probability of mortality during the TTD assay of animals previously exposed 0.5 mg/L versus 1 mg/L of carbaryl. Here a hazard ratio  $< 0$  indicates a decrease in the probability of mortality of

the animals previously exposed to 0.5 mg/L relative to 1 mg/L or carbaryl; a hazard ratio > 0 indicates the reverse outcome.

## **5.3 RESULTS**

### **5.3.1 Hopscotch Pond**

Relative to embryonic exposure to 0 mg/L, the Cox regression analysis found that embryonic exposure to both 0.5 and 1 mg/L of carbaryl induced higher tolerance to a lethal dose of carbaryl as tadpoles (both  $p < 0.001$ ). Hazard ratios indicate that tadpoles exposed to 0.5 and 1 mg/L of carbaryl were both more tolerant to a lethal concentration of carbaryl than tadpoles that were not exposed to carbaryl as embryos (Fig. 1; Table 2). When comparing TTD of tadpoles exposed to 0.5 vs. 1 mg/L of carbaryl as embryos we found no difference ( $p = 0.47$ ; Table 2).

We then examined two insecticides that share the same mode of action with carbaryl: chlorpyrifos and malathion. Using chlorpyrifos, embryonic exposure to 0.5 and 1 mg/L of carbaryl did not significantly affect tadpole TTD compared to embryonic exposures to 0 mg/L of carbaryl ( $p = 0.84$ ;  $p = 0.52$ ). Using malathion, embryonic exposures to 0.5 mg/L did not significantly affect tadpole TTD when exposed to a lethal concentration of malathion ( $p = 0.27$ ) compared to embryonic exposures to 0 mg/L of carbaryl. However, embryonic exposure to 1 mg/L of carbaryl increased tadpole TTD when exposed to a lethal concentration of malathion compared to embryonic exposures to 0 mg/L of carbaryl ( $p = 0.03$ ; Fig. 1; Table 2). When comparing TTD of tadpoles exposed to 0.5 vs. 1 mg/L of carbaryl as embryos we found no significant effect of chlorpyrifos ( $p = 0.22$ ) or malathion ( $p = 0.32$ ; Table 2).

We also examined two insecticides that have different modes of action than carbaryl: permethrin and cypermethrin. Using permethrin, we found that embryonic exposure to 0.5 and 1 mg/L concentrations of carbaryl did not affect tadpole TTD when exposed to a lethal concentration of permethrin ( $p = 0.77$ ;  $p = 0.17$ , respectively) compared to tadpoles that received an embryonic exposure to 0 mg/L of carbaryl. Using cypermethrin, embryonic exposure to 0.5 mg/L of carbaryl did not affect tadpole TTD when exposed to a lethal concentration of cypermethrin compared to tadpole given an embryonic exposure to 0 mg/L of carbaryl ( $p = 0.14$ ). However, embryonic exposure to 1 mg/L of carbaryl did affect tadpole TTD when exposed to a lethal concentration of cypermethrin ( $p = 0.006$ ). The hazard ratio analysis indicated that tadpoles exposed to 1 mg/L of carbaryl as embryos were less tolerant to a lethal concentration of cypermethrin compared to tadpoles exposed to 0 mg/L of carbaryl (Fig. 1; Table 2). Finally, when comparing TTD of tadpoles embryonically exposed to 0.5 vs. 1 mg/L of carbaryl, we found no difference when tadpoles were exposed to permethrin ( $p = 0.13$ ) but there was a difference when tadpoles were exposed to cypermethrin ( $p < 0.001$ ). Hazard ratio analysis indicated that tadpoles exposed to 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of cypermethrin than tadpoles exposed to 1 mg/L of carbaryl as embryos (Table 2).

### **5.3.2 Square Pond**

Compared to embryonic exposures to 0 mg/L, embryonic exposure to both 0.5 and 1 mg/L of carbaryl induced higher tolerance to lethal concentrations of carbaryl as tadpoles ( $p < 0.001$ ;  $p = 0.001$ , respectively). Similar to Hopscotch Pond, hazard ratios indicated that tadpoles exposed to 0.5 and 1 mg/L of carbaryl were more tolerant to a lethal concentration of carbaryl than tadpoles that were not exposed to carbaryl as embryos (Fig. 2; Table 3). When comparing TTD of

tadpoles exposed to 0.5 vs. 1 mg/L of carbaryl as embryos we found no significant effect ( $p = 0.25$ ; Table 3).

For insecticides that share the same mode of action with carbaryl, the Cox regression analysis found that embryonic exposure to 0.5 and 1 mg/L of carbaryl did not affect TTD relative to embryonic exposure to 0 mg/L when exposed to a lethal concentration of chlorpyrifos ( $p = 0.06$ ;  $p = 0.48$ ) or malathion ( $p = 0.45$ ;  $p = 0.13$ ). When we compared TTD of tadpoles exposed to 0.5 vs. 1 mg/L of carbaryl as embryos, we found no effect of embryonic exposures with chlorpyrifos ( $p = 0.26$ ) but there was an effect of embryonic exposures with malathion ( $p = 0.03$ ; Fig. 2; Table 3). Hazard ratios indicated that tadpoles exposed 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of malathion compared to tadpoles exposed to 1 mg/L carbaryl as embryos.

For insecticides that have different modes of action than carbaryl, the Cox regression analysis found that embryonic exposure to 0.5 and 1 mg/L concentrations of carbaryl did not significantly affect TTD when exposed to a lethal concentration of permethrin ( $p = 0.13$ ;  $p = 0.61$ , respectively). In contrast, embryonic exposure to both 0.5 and 1 mg/L of carbaryl significantly affected tadpole TTD when exposed to a lethal concentration of cypermethrin ( $p = 0.045$ ;  $p = 0.018$ , respectively). The analysis of hazard ratios indicated that tadpoles exposed to 0.5 mg/L of carbaryl as embryos were significantly more tolerant to a lethal concentration of cypermethrin compared to tadpoles with no previous exposure (Fig. 2; Table 3). However, similar to Hopscotch Pond, tadpoles exposed to 1 mg/L of carbaryl as embryos became less tolerant to a lethal concentration of cypermethrin compared to tadpoles not exposed to carbaryl as embryos (i.e. a hormetic dose response). (Calabrese and Baldwin 2003) Finally, when comparing TTD of tadpoles exposed to 0.5 vs. 1 mg/L of carbaryl as embryos, we found a no

effect with permethrin ( $p = 0.33$ ) but there was an effect with cypermethrin ( $p < 0.001$ ). Hazard ratio analysis indicated that tadpoles from Square Pond exposed to 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of cypermethrin than tadpoles exposed to 1 mg/L of carbaryl as embryos (Table 3).

## 5.4 DISCUSSION

Similar to the first study that discovered that sublethal exposure to carbaryl induced increased tolerance to carbaryl in wood frogs (Hua et al. 2013b), this study also demonstrated that embryonic exposure to sublethal concentrations of carbaryl induced increased wood frog tadpole tolerance to carbaryl. This was a necessary first step in testing for cross-tolerance. For tadpoles from Hopscotch Pond, the sublethal concentrations that induced tolerance (0.5 and 1 mg/L) to a subsequent lethal dose of carbaryl were consistent with the results of Hua et al. (2013b). In the current study, tadpoles from Square Pond exposed to 0.5 and 1 mg/L of carbaryl as embryos both exhibited higher tolerance to a lethal dose of carbaryl. In contrast, Hua et al. found that while 0.5 mg/L of carbaryl induced higher tolerance, 1 mg/L of carbaryl induced lower tolerance to carbaryl (Hua et al. 2013b). Despite the variation in the concentration of carbaryl that induces tolerance, we show that the general phenomenon of induced higher tolerance is repeatable.

Given that an early exposure to carbaryl can induce higher tolerance to a lethal exposure to carbaryl, we hypothesized that it might also induce cross-tolerance to insecticides that share the same mode of action (i.e. malathion and chlorpyrifos). Indeed, sublethal exposures to carbaryl induced a higher tolerance to a lethal concentration of malathion. In a recent study we found that populations of wood frog tadpoles that were not exposed in the laboratory to an early

sublethal concentration of carbaryl but were constitutively tolerant to carbaryl were also constitutively cross-tolerant to malathion (Hua et al. 2013a). However, the current study is the first to demonstrate that cross-tolerance can also occur through the process of induction.

The pattern of induced cross-tolerance to malathion differed between the two populations. Embryonic exposure to 1 mg/L carbaryl induced cross-tolerance to malathion in tadpoles from Hopscotch Pond but not in tadpoles from Square Pond. Due to a number of factors including population history of insecticide exposure, wood frog populations can vary in their constitutive and induced tolerance (Bridges and Semlitsch 2000; Cothran et al. 2013; Hua et al. 2013b). To understand the contribution of cross-tolerance in buffering non-target organisms from chemical contaminants, future studies expanding upon the population-level factors that drive this phenomenon are an important next step.

Despite the fact that chlorpyrifos shares a similar mode of action with carbaryl, we found no evidence that sublethal embryonic exposure to carbaryl induced cross-tolerance to chlorpyrifos. The TTD of tadpoles from all Phase 1 treatments exposed to lethal concentrations of chlorpyrifos was 73% and 53% earlier than the TTD of tadpoles exposed to lethal concentrations of carbaryl for Hopscotch and Square Ponds, respectively. These lower TTD values suggest that the concentration of chlorpyrifos used in our TTD assay may have been too high to allow the observation of induced tolerance. As noted earlier, the objective of a TTD assay is to cause some mortality but not immediate mortality (Newman 2010). Exposure to higher concentrations can overwhelm the ability of organisms to tolerate the insecticide (Georghiou 1990; Oakeshott et al. 2005b). Thus, future studies using lower concentrations of chlorpyrifos would be helpful in more fully examining whether induced cross-tolerance occurs with chlorpyrifos.

For the two insecticides that have a different mode of action than carbaryl, we hypothesized that embryonic exposure to sublethal carbaryl would not induce higher cross-tolerance. Surprisingly, for tadpoles from Square pond, we found that embryonic exposure to 0.5 mg/L of sublethal carbaryl induced higher tolerance to cypermethrin. Although not statistically significant, tadpoles from Hopscotch Pond followed a similar pattern with tadpoles exposed to 0.5 mg/L of carbaryl having the highest tolerance to cypermethrin. Although constitutive cross-tolerance to insecticides with different modes of action has been documented in several targeted pest species (Liu and Yue 2000; Alou et al. 2010), this is the first study to demonstrate that induced cross-tolerance to insecticides can occur for insecticides with different modes of action.

The process of induced cross-tolerance, which happens within just a few days, has critical implications for non-target species population persistence. In particular, amphibian populations are declining worldwide for a number of reasons, including a hypothesized link to pesticides (Faust et al. 2000; Sparling and Fellers 2009; Wake 2012). With growing human populations and increased dependence on pesticides (Laurance 2001), the ability to induce higher tolerance and cross-tolerance has significant conservation implications for the persistence of populations unintentionally exposed to pesticides. Although TTD assays examine differences in pesticide tolerance by measuring differences in survival when exposed to a high pesticide concentration over a few days, the differences in tolerance are likely to also be expressed in other traits at lower concentrations. As a result, we can hypothesize that tadpoles induced to have higher tolerance to an insecticide (as measured by survival) would also have improved performance (e.g., less impaired behaviors) when subsequently exposed to low concentrations of the insecticide. Collectively, knowledge of induced tolerance and cross-tolerance may alter how we think about and manage insecticide tolerance in target and non-target species alike.



The induction of cross-tolerance to cypermethrin in tadpoles from Square Pond did not follow a typical dose-dependent pattern. Instead, sublethal concentrations exhibited a nonlinear hormetic response. A hormetic response occurs when exposure to a low concentration of a chemical agent or environmental factor is beneficial to an individual but a higher concentration is damaging to the individual (Hayes et al. 2002; Mattson 2008). Such responses are commonly found in response to environmental toxins (Costantini et al. 2010). In this study, tadpoles from Square Pond exposed to the 0.5 mg/L of carbaryl as embryos induced a higher tolerance to cypermethrin while tadpoles exposed to 1 mg/L of carbaryl as embryos induced a lower tolerance to cypermethrin. For Hopscotch pond, though not statistically significant, the classic inverted “J” pattern of tadpole tolerance exposed to 0, 0.5, and 1 mg/L of carbaryl as embryos (low concentration inducing highest tolerance, control concentration inducing intermediate tolerance, and high concentration inducing lowest tolerance) is suggestive of a hormetic pattern (Calabrese and Baldwin 2003). Collectively, these results indicate that the concentration of insecticide inducing higher cross-tolerance may be confined to a narrow range for cypermethrin. An important challenge towards understanding the phenomenon of induced cross-tolerance is to identify the range of concentrations that lead to the induction of increased versus decreased pesticide tolerance.

Finally, although exposure to 0.5 mg/L carbaryl induced higher tolerance to cypermethrin, we found no evidence of induced cross-tolerance to permethrin, which has the same mode of action as cypermethrin. However, only 7% to 24% of tadpoles from the two populations died from exposure to permethrin. Such low mortality makes it difficult to reliably assess whether prior exposures to carbaryl affect the subsequent tolerance to permethrin. To determine the possibility of induced cross-tolerance to permethrin, future studies should use

higher concentrations of permethrin to more accurately assess differences in tolerance later in life caused by exposure to different sublethal treatments early in life.

In summary, the goal of this study was to investigate whether embryonic exposure to sublethal concentrations of one insecticide can induce cross-tolerance to other insecticides that have the same or different modes of actions. This is the first study to demonstrate the induction of cross-tolerance. Further, we show that induced cross-tolerance is not just limited to insecticides of the same mode of action. The patterns of induced cross-tolerance varied across the two populations, which highlight the need for future studies to consider the factors driving population-level variation in the inducibility of tolerance. Finally, there was one case where sublethal concentrations of carbaryl initiated a nonlinear hormetic dose response, demonstrating that the inducibility of cross-tolerance may be confined to a narrow range of concentrations for some pesticides. The rapid process of the induction of higher tolerance and cross-tolerance has promising implications for the persistence of amphibian populations in the face of chemical contaminants.

With the discovery of induced higher tolerance and cross-tolerance, the critical next step is to determine the relative contribution of this phenomenon to the persistence of non-target species exposed to insecticides. Toward this goal, we need to (1) determine the generalizability of induced tolerance across different taxa, (2) pinpoint the biotic and abiotic factors that may lead to the induction of increased versus decreased tolerance, (3) investigate the length of time that induced tolerance is retained, and (4) identify potential trade-offs associated with inducing increased tolerance. Further, this study uses survival as an indicator of population persistence, future studies should also explore other indicators of post-metamorphic fitness (i.e. mass at and time to metamorphosis) as these factors have also been shown to affect amphibian population

persistence. Thus, future studies should consider whether population induced to have higher tolerance for survival also have higher tolerance in other measures of performance. Addressing these factors will not only aid in conservation efforts for non-target populations but also have broad multidisciplinary applications to the understanding of ecological and evolutionary mechanisms shaping patterns of species abundances in response to anthropogenic contaminants.

**Table 5-1. Table 1. Chemical name (common formulated product), class and mode of action, and concentrations found in natural bodies of water of carbaryl, chlorpyrifos, malathion, permethrin, and cypermethrin.**

Insecticide	Chemical class; mode of action	Observed environmental concentrations
Carbaryl (Sevin ®)	Carbamate; AChE inhibitor	0.73- 1.5 mg/L <sup>1</sup>
Chlorpyrifos	Organophosphate; AChE inhibitor	0.00031- 0.025 mg/L <sup>2</sup>
Malathion (Malathion Plus 50%)	Organophosphate; AChE inhibitor	0.029 - 0.583 mg/L <sup>3,4</sup>
Cypermethrin (Enforcer ® Overnite)	Pyrethroid <sup>3</sup> ; Na <sup>+</sup> channel interference	0.002 - 0.03 mg/L <sup>5,6</sup>
Permethrin (ORTHO ® Basic)	Pyrethroid; Na <sup>+</sup> channel interference	0.000094- 3.114 mg/L <sup>7,8</sup>

<sup>1</sup> <http://www.inchem.org/documents/ehc/ehc/ehc153.htm>

<sup>2</sup> [http://www.apvma.gov.au/products/review/docs/chlorpyrifos\\_env.pdf](http://www.apvma.gov.au/products/review/docs/chlorpyrifos_env.pdf)

<sup>3</sup> Newhart, K.L 2006. Environmental fate of malathion. California Environmental Protection Agency, Department of Pesticide Regulation (Environmental Monitoring Branch).

<sup>4</sup> Nalini, S., K.S. Ajit, R. Sanghi. 2005. Organochlorine and organophosphorus pesticide residues in ground waters of Kanpur, Uttar Pradesh, India. Environment International 31: 113-120.

<sup>5</sup> Due to the physical characteristics of pyrethroids, their tendency to sorb to suspended sediments (organic carbons), and the low concentrations detected, few studies have measurable concentrations may not be detected in a larger river system such as the Sacramento or San Joaquin River.”<http://www.cdpr.ca.gov/docs/emon/pubs/ehapreps/eh0401.pdf>

<sup>6</sup> Defra Science Directorate. 2007. Environmental exposure to cypermethrin released from farmyard. Report No. VM02502. Central Science Laboratory, York, UK.

<sup>7</sup> Bacey, J., K. Starner, and F. Spurlock. 2004. The Occurrence and Concentration of Esfenvalerate and Permethrin in Water and Sediment in the Sacramento and San Joaquin Watersheds. Sacramento, CA: CA Environmental Protection Agency.

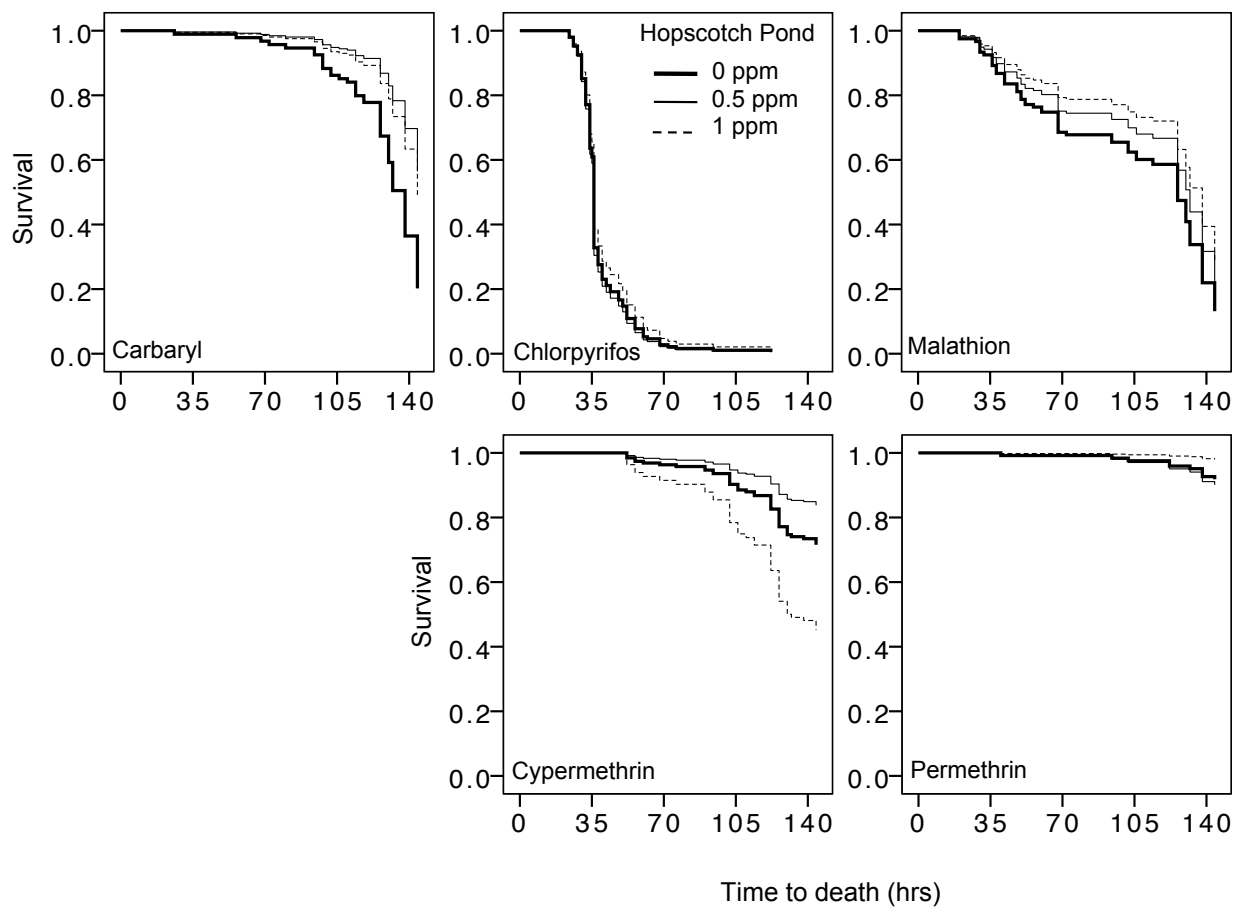
<sup>8</sup> Bishop, W.E. 1983. Aquatic Toxicology and Hazard Assessment: 6<sup>th</sup> Symposium. ASTM International. West Conshohocken, PA.

**Table 5-2. Hazard ratios for tadpoles from Hopscotch Pond that had been previously exposed to three sublethal concentrations of carbaryl as embryos (0, 0.5 or 1.0 mg/L) and then exposed as tadpoles to a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin. In each comparison, a negative hazard ratio indicates that the first embryonic concentration made tadpoles less tolerant than the second embryonic concentration. A positive hazard ratio indicates the opposite phenomenon.**

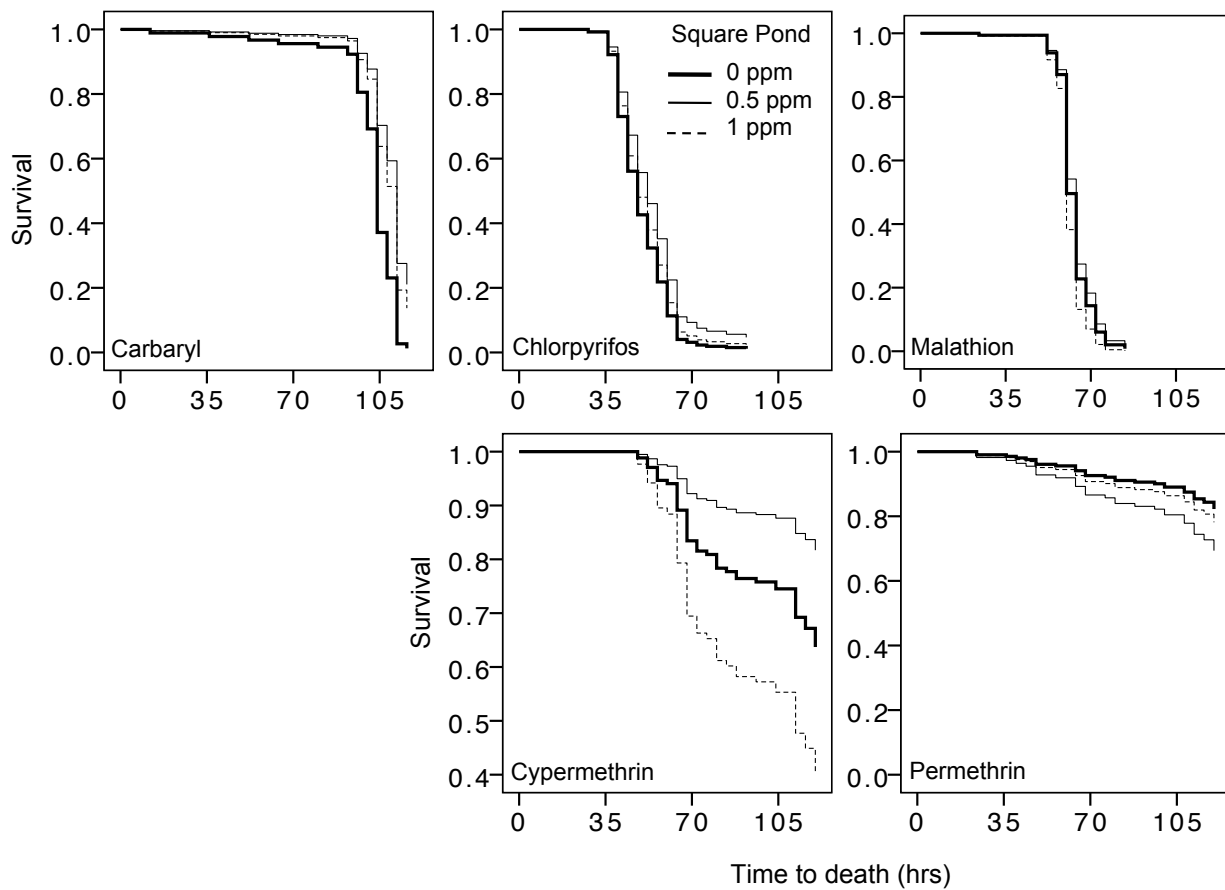
Insecticide	Embryonic carbaryl exposure hazard ratios; Percent censored (p-value)		
	0 mg/L vs. 0.5 mg/L	0 mg/L vs. 1 mg/L	0.5 mg/L vs. 1 mg/L
Carbaryl	-1.02; 41% <b>(&lt;0.001)</b>	-0.77; 35% <b>(0.002)</b>	0.22; 55% (0.47)
Chlorpyrifos	0.04; 0.7% (0.84)	-0.13; 0% (0.52)	-0.25; 1% (0.22)
Malathion	-0.24; 11% (0.27)	-0.5; 20% <b>(0.03)</b>	0.23; 25% (0.31)
Cypermethrin	-0.64; 77% (0.15)	0.86; 59% <b>(0.008)</b>	1.5; 65% <b>(&lt;0.001)</b>
Permethrin	0.2; 91% (0.77)	-1.4; 95% (0.2)	-1.65; 92% (0.13)

**Table 5-3. Hazard ratios for tadpoles from Square Pond that had been previously exposed to three sublethal concentrations of carbaryl as embryos (0, 0.5 or 1.0 mg/L) and then re-exposed as tadpoles to a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, and permethrin. In each comparison, a negative hazard ratio indicates that the first embryonic concentration made tadpoles less tolerant than the second embryonic concentration. A positive hazard ratio indicates the opposite phenomenon.**

Insecticide	Embryonic carbaryl exposure hazard ratios; Percent censored (p-value)		
	0 mg/L vs. 0.5 mg/L	0 mg/L vs. 1 mg/L	0.5 mg/L vs. 1 mg/L
Carbaryl	-1.06; 11% <b>(&lt;0.001)</b>	-0.72; 9% <b>(0.001)</b>	0.26; 20% (0.25)
Chlorpyrifos	-0.04; 2.9% (0.06)	-0.14; 2% (0.48)	0.23; 3% (0.26)
Malathion	-0.15; 0% (0.45)	0.31; 0% (0.13)	0.46; 0% <b>(0.03)</b>
Cypermethrin	-0.8; 73% <b>(0.05)</b>	0.69; 52% <b>(0.02)</b>	1.5; 61% <b>(&lt;0.001)</b>
Permethrin	0.63; 76% (0.14)	0.23; 80% (0.61)	-.39; 74% (0.33)



**Figure 5-1. The survival of wood frog tadpoles from Hopscotch Pond over time after being exposed to different sublethal concentrations of carbaryl as embryos and a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin as tadpoles.**



**Figure 5-2. The survival of wood frog tadpoles from Square Pond over time after being exposed to different sublethal concentrations of carbaryl as embryos and a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin as tadpoles.**



## **6.0 CONCLUSIONS**

As human populations continue to grow, understanding the unintentional consequences of pesticides in natural systems has significant implications. However, traditional studies of toxicology are not always sufficient in predicting the effects of pesticides in complex community scenarios (Fleeger et al. 2003). To address this limitation, Chapter 2 of this thesis tracked the direct and indirect effects of four common insecticides and a mixture across multiple trophic level for 18 weeks. This study demonstrated that insecticides applied individually and in a mixture have complex direct and indirect consequences on aquatic system response and recovery not predicted by traditional laboratory tests. However, despite these unique direct and indirect effects, this study demonstrates that by considering the ecological interaction between taxa and temporal variation in life history, generalizable predictions can be made about the effects of these insecticides even in complex aquatic communities.

While this study incorporated several added factors of ecological complexity (i.e. multiple trophic levels, temporal complexity; pesticide mixtures), future studies incorporating step-wise approaches toward integrating more ecological complexity are critical. For instance, this mesocosm study contained over 20 animal taxa across multiple trophic levels, but we did not included secondary consumers which have been shown to interact with pesticide toxicity (Relyea and Mills 2001). Future studies continuing to incorporate additional levels of trophic complexity are needed. This study also only examine one mixture combination– future studies should

examine other mixtures with the ultimate goal of developing our ability to predict situations in which additive, synergistic, or antagonistic scenarios of mixtures occur. Finally, though mesocosms are ideal venues for controlled studies of the effects of pesticides on whole communities, future studies in natural aquatic systems will be necessary.

Insecticide tolerance and cross-tolerance in non-target organisms is often overlooked despite its potential to buffer natural systems from anthropogenic influence. In Chapter 3, this thesis demonstrates that amphibian populations with tolerance to one pesticide may be tolerant to many other pesticides. Cross-tolerance to insecticides should reduce the episodes of selection experienced by populations and thus minimize both negative pleiotropic effects and eliminate the erosion of genetic variation in the population (Coors et al. 2009). Our results suggest that amphibians with cross-tolerance are not only more tolerant to the insecticide causing selection, but may also be tolerant to a wide range of similarly acting insecticides. With amphibians worldwide experiencing unprecedented declines and some of these declines being associated with insecticide use (Davidson 2004), future studies quantifying the existence and prevalence of cross-tolerance towards contributing significant conservation insights.

In rapidly changing environmental conditions, the contribution of plasticity has critical implications for individuals and the evolution of populations by allowing adaptive traits to be induced rapidly within a single generation. Yet, this phenomenon has only been considered in one other species (Poupardin et al. 2008b; Poupardin et al. 2012). The study described in Chapter 4 is the first to demonstrate that sublethal and ecologically relevant concentrations of a common insecticide can, within the same generation, induce adaptive tolerance in a vertebrate species. Further, this study demonstrated that the population-level pattern of inducibility is consistent with predictions of genetic assimilation. To sum, inducible responses to anthropogenic

disturbances may have a significant impact on shaping patterns of species abundance. Future work identifying the underlying mechanisms that drive these inducible responses to insecticides as well as other anthropogenic contaminants are an important step towards understanding the long-term impact of disturbances on natural systems. The role of plasticity in shaping adaptations is important to understanding the expression of traits within individuals and the evolution of populations.

Finally, given the enormity of available pesticides (USEPA 2007) induced pesticide tolerance would be particularly beneficial to non-target species if it were to confer increased tolerance not only against the pesticide it first experienced, but also against many other pesticides (e.g., induced cross-tolerance). The study described in Chapter 5, is the first to demonstrate the phenomenon of induced cross-tolerance. Further, this study found that exposure to sublethal pesticides can induce tolerance to not only single pesticides but can induce cross-tolerance to multiple pesticides.

With the discovery of induced higher tolerance and cross-tolerance (Chapter 4 and 5), the critical next step is to determine the relative contribution of this phenomenon to the persistence of non-target species exposed to insecticides. Toward this goal, future studies need to determine the generalizability of induced tolerance across different taxa, identify the biotic and abiotic factors that may lead to the induction of increased versus decreased tolerance, determine the length of time that induced tolerance is retained, and identify potential trade-offs associated with inducing increased tolerance. Further, both induced tolerance studies used survival as an indicator population persistence, future studies should also explore other indicators of post-metamorphic fitness (i.e. mass at and time to metamorphosis) as these factors have also been shown to affect amphibian population persistence. Thus, future studies should consider whether

population induced to have higher tolerance for survival also have higher tolerance in other measures of performance. Addressing these factors will not only aid in conservation efforts for non-target populations but also have broad multidisciplinary applications to the understanding of ecological and evolutionary mechanisms shaping patterns of species abundances in response to anthropogenic contaminants. To sum, by using disturbances to perturb natural systems, biologist can both develop generalizable predictions about how disturbances alter natural systems as well as utilize disturbances as a tool to test ecological and evolutionary theory.

## **APPENDIX A**

### **ADDITIONAL INFORMATION (CHAPTER 2)**

#### **A.1.1 Mesocosm set up- Pond water and zooplankton additions**

We collected pond water from three nearby ponds and visually screened the water for invertebrate predators. After removing the predators, we added equal aliquots (354 ml) to each mesocosm to provide a natural source of algae and bacteria. We vertically placed four unglazed ceramic tiles (15 x 15 cm) on the north side of each mesocosm to serve as periphyton samplers. On 20 April and 1 May, we collected zooplankton from four local ponds using a 30-micron zooplankton tow. After removing any predators from this sample we combined the zooplankton from the four populations and added equal aliquots of the zooplankton/ pond water mix to each mesocosm.

#### **A.1.2 Addition of animals to mesocosms**

For the snails, we collected 100 adults of each species (*Physa acuta*, *Helisoma trivolvis*, and *Stagnicola elodes*) from three local ponds and wetlands. To avoid the introduction of parasites, we placed groups of five conspecific snails in individual cups with 500 ml of water and fed them

rabbit chow ad libitum. Once a cup had received five egg masses, the adults were removed. One cup from each snail species was then suspended in the water column of each mesocosm and allowed to hatch. Once eggs hatched, we removed the cups.

### **A.1.3 Confirmation of nominal insecticide concentration**

Within four hours after dosing the mesocosms, we collected ~0.89 L of water from each of the mesocosms, pooled the samples within a given pesticide treatment, and sent the samples to Mississippi State Chemical Laboratory for high-pressure liquid chromatography analysis (lower detection limit = 0.1 ug/L). Controls were negative for all insecticides except for malathion, which had trace amounts (0.2 ug/L). Independent studies conducted in close proximity to our study also found trace amounts of malathion in control samples suggesting that the malathion detected in this study in the control was not experimental error but perhaps due to contamination of source water or errors in the chemical analytical process (Brogan and Relyea, in review; Groner et al., 2013).

Relative to the nominal concentrations of 10 and 40 ug/L, the actual concentrations of each insecticide were as follows: chlorpyrifos = 2.7 ug/L and 21 ug/L; diazinon = 6.7 ug/L and 34 ug/L; endosulfan = 0.66 ug/L and 5.2 ug/L; malathion = 2.8 ug/L and 12 ug/L. We did not test the concentrations in the mixture treatment sample since they were made from the same concentrations of chemicals used to dose each of the 10 ug/L individual insecticide treatments. Based on these measured concentrations, the total concentration of the mixture would be approximately 13 ug/L. After adding the summer-breeding species of amphibians (3 wks after the single insecticide application), we collected another water sample for analysis. For the nominal concentrations of 10 and 40 ug/L, respectively, we detected 0.8 ug/L and 0.6 ug/L of

chlorpyrifos, 4.1 ug/L and 16 ug/L of diazinon, and no detectible concentration of malathion or endosulfan. Our water sample indicates that the insecticides are no longer in the water column. However, it is possible that the insecticides have sorbed into organic materials or have broken down into more toxic compounds. Future studies are needed to address these possibilities.

Despite storing samples in accordance to established analytical methods (OECD 2007), the actual concentrations we detected were lower than nominal concentrations. Decades of community experiments in mesocosms suggests that lower actual concentrations are common and can be due to sample degradation through a variety of biological and chemical processes (Sherma and Beroza 1980; Rohr and Crumrine 2005; Buck et al. 2012; Relyea 2012b). Since the first and second sets of samples were processed two weeks and five weeks after collection, it is possible that these samples experienced some degree of degradation. Thus, given that the amount of pesticides added to each tank was correctly calculated, we are confident that these lower concentrations are due to storage degradation rather than an error in pesticide application. In this paper, we will refer to the actual concentrations detected but for simplicity, we will denote nominal concentration of 10 ug/L as the “low concentration” and 40 ug/L as the “high concentration.”

#### **A.1.4 Quantifying light attenuation**

Light attenuation was measured as the rate of sunlight decay quantified in each mesocosm using an underwater light meter (LI-COR, Lincoln, Nebraska, USA). We measured photosynthetically active radiation at depths of 10 cm and 30 cm during weeks 2, 4, 9, and 18. The decay rate of light with increased water depth (K) was determined using the following formula:

$$K = \frac{\ln (L10/L30)}{d}$$

Where L10 is the intensity of sunlight at 10 cm from the surface, L30 is the intensity of sunlight at a depth of 30 cm, and d refers to the difference in depth between the two intensity measurements.

#### **A.1.5 Zooplankton sampling**

Zooplankton assemblages were sampled from five locations in the water column for each mesocosm during weeks 2, 4, 9, and 18 using a 0.2-L tube sampler. The five samples were filtered through a 30-micron nitex net and preserved in 30% ethanol. All zooplankton samples were identified to species. We found that the zooplankton assemblage was dominated (> 90%) by two species of cladocerans (*Daphnia pulex* and *Daphnia magna*) and one species of copepod (*Microcyclops rubellus*). The remaining species were relatively rare: *Ceriodaphnia lacustris* (<1%), *Daphnia longiremus* (<1%), *Daphnia galeata mendotae* (<1%), *Scapheloberis mucronata* (3%) and *Chydorus sphaericus* (5%), *Skistodiaptomus oregonensis* (<1%). Although cladocerans and copepods typically have different sensitivities to insecticides, different species of cladocerans all respond very similarly to a given insecticide and different species of copepods respond very similarly to a given insecticide (Hughes et al 1980; Chang et al 2005; Relyea 2005). As a result, we analyzed the zooplankton using species that were pooled as either cladocerans or copepods.



#### **A.1.6 Phytoplankton sampling**

Phytoplankton samples were filtered using GF/ C47 mm Whatman glass microfiber filters (Whatman, Incorporated, Florham Park, New Jersey, USA). To prevent sample degradation, each sample was vacuum filtered, wrapped in aluminum foil and then frozen to -29°C. The frozen samples were later analyzed for chlorophyll a concentrations following the protocols developed by Arar and Collins (1997) between 5 September and 31 October 2009. We used a fluorometer (Model ED-700, Turner Designs, Sunnyvale, California, USA) to assay the chlorophyll a concentrations.

#### **A.1.7 Collecting amphibian metamorphs**

We began collecting metamorphic wood frogs and American toads during week 4, spring peepers during week 5, leopard frogs during week 6, and gray treefrogs during week 8. Metamorphs were removed as soon as all four limbs emerged and tail length was < 4 cm. After capture, metamorphs were placed in covered plastic containers filled with moist sphagnum until complete tail re-absorption (Gosner stage 46, Gosner 1960). Complete metamorphs were then euthanized using a 3% MS-222 solution and preserved in a 10% solution of buffered formalin.

#### **A.1.8 Response and recovery of the abiotic variables**

In our analysis of the four abiotic factors, we found significant multivariate effects of pesticides and pesticides-by-time interactions but no significant effect of time (Table A3). The univariate tests indicated significant pesticide effects on pH, dissolved oxygen, and light attenuation and

significant pesticides-by-time interactions for pH and light attenuation (Figures A1-A4). To understand these ecosystem level effects, we first examined how each abiotic variable was affected by individual insecticides compared to the control then compared these responses to the pesticide mixture.

#### **A.1.9 pH.**

At the low concentration, pH showed response to malathion and endosulfan while chlorpyrifos and diazinon caused an increase in pH at week 9 (Figure A2). However, the pH showed recovery in these treatments, bouncing back to control levels by week 18. At the high concentration, pH showed response to malathion but endosulfan, diazinon, and chlorpyrifos caused an increase in pH at week 4. The pH exhibited recovery in the endosulfan, diazinon and chlorpyrifos treatments by week 18. The mixture of all four insecticides caused an increase in pH at week 4, but pH was once again resilient by week 18. The effect of the mixture on pH was similar to the low concentration of endosulfan, diazinon, and chlorpyrifos but exceeded the effects of the low concentration of malathion. The effect of the mixture on pH was similar to the high concentration of diazinon and chlorpyrifos, but was higher than the effects of malathion, but was lower than the effects of endosulfan.

#### **A.1.10 Dissolved oxygen**

At the low concentration, dissolved oxygen was resistant to malathion, chlorpyrifos, and endosulfan but diazinon caused an increase at week 9 (Figure A3). Dissolved oxygen showed recovery to diazinon, bouncing back by week 18. At the high concentration, dissolved oxygen

was resistant to malathion, diazinon, and chlorpyrifos but endosulfan caused an increase in dissolved oxygen at week 4. Dissolved oxygen showed recovery to endosulfan, bouncing back by week 18. The mixture of all four insecticides had no effect on dissolved oxygen compared to the control, but at week 2 the effect of the mixture on dissolved oxygen exceeded the effect of malathion at the high concentration.

#### **A.1.11 Light attenuation**

Light attenuation was resistant to exposure to the low concentrations of malathion and endosulfan while chlorpyrifos and diazinon caused increased light attenuation at week 9 (Figure A4). Light attenuation in the chlorpyrifos treatment exhibited recovery by week 18 but it never recovered to control levels in the diazinon treatment. Light attenuation was resistant to the high concentrations of malathion but diazinon, chlorpyrifos, and endosulfan caused an increase in light attenuation at week 9. Light attenuation exhibited recovery in all of these insecticide treatments by week 18. Light attenuation was resistant to the mixture of all four insecticides, with effects that were similar to each insecticide at both the low and high insecticide treatment concentrations.

#### **2.2. Response and recovery of cladocerans, copepods, phytoplankton, and periphyton**

In our analysis of cladocerans, copepods, phytoplankton, and periphyton abundance, we found significant multivariate effects of pesticide, time, and the pesticide-by-time interactions (Table A4). The univariate tests indicated that periphyton abundance was only affected by time, but cladocerans, copepods, and phytoplankton abundance were affected by pesticides and the pesticides-by-time interaction. As a result, we assessed the effect of pesticides for each variable within each sample time point.

#### **A.1.12 Cladocerans**

The abundance of cladocerans was affected by the pesticide treatment at every time point (Table A4C). At week 2, cladocerans were resistant to the low concentration malathion while the low concentrations of chlorpyrifos and diazinon reduced abundance and endosulfan increased abundance (Fig. 1). Cladocerans exposed to the low concentration of chlorpyrifos and diazinon were resilient by week 18 and those exposed to endosulfan were resilient by week 4. Cladocerans experienced immediate reductions in abundance when exposed to the high concentration of malathion, diazinon, and chlorpyrifos whereas the high concentration of endosulfan caused a reduction midway in the experiment. Following exposure to these high concentrations, cladocerans rebounded to control levels 9 weeks after malathion was added, 18 wks after diazinon and endosulfan were added, and they never rebounded after chlorpyrifos was added. The mixture of all four insecticides caused a large reduction in cladocerans that ultimately exceeded the effects of any individual insecticide at the low concentration and exceeded the effects of malathion and endosulfan at the high concentration. Cladocerans in the insecticide mixture never rebounded to control levels thus were similarly resilient to chlorpyrifos at the high concentration but were less resilient compared to both the low and high concentrations of all other insecticide treatments.

#### **A.1.13 Copepods**

The abundance of copepods was affected by the pesticide treatment at every time point (Table A4C). At the low concentration, copepods were resistant to malathion while endosulfan, diazinon and chlorpyrifos caused a significant decrease in abundance at week 2 (Fig. 1).

Copepods exposed to the low concentration of endosulfan and diazinon were resilient and bounced back to control abundances by week 4. Copepod abundances exposed to the low concentration of chlorpyrifos recovered briefly at week 9 but was significantly reduced again at week 18. At the high concentration, copepods were resistant to diazinon but experienced reductions in abundance with endosulfan, malathion, and chlorpyrifos at week 2. Following exposure to endosulfan, copepod abundances remained low throughout the experiment but were only significantly lower than control abundances at weeks 9 and 18. For chlorpyrifos, copepod abundances remained low and did not recover to control abundances prior to the end of the study. The mixture of all four insecticides caused an immediate reduction in copepod abundance that was similar to the individual effects of endosulfan, diazinon, and chlorpyrifos at the low concentration but exceeded the individual effects of the low concentration of malathion. Compared to individual applications of the high concentration, the mixture effect on copepod response was similar to endosulfan and chlorpyrifos but exceeded the effects of malathion and diazinon. Copepod abundance in the insecticide mixture recovered to control levels at week 18 thus was less resilient compared to all insecticide treatments but low and high chlorpyrifos and high endosulfan.

#### **A.1.14 Phytoplankton**

The abundance of phytoplankton was affected by the pesticide treatment at weeks 4, 9, and 18 (Table A4). At the low concentration, phytoplankton was resistant to applications of endosulfan and malathion while diazinon and chlorpyrifos caused an increase in phytoplankton at week 4 and 9 (Fig. 2). Phytoplankton abundance in the low concentration diazinon and chlorpyrifos was resilient by week 18. At the high concentration, phytoplankton abundance decreased with

endosulfan (wk 4) but increased with chlorpyrifos (wk 18) and diazinon (wk 9). Following the high concentration exposures, phytoplankton rebounded to control levels 9 wks after endosulfan was added but did not recover prior to the end of the experiment following exposure to chlorpyrifos and diazinon. The mixture of all four insecticides caused a significant increase in phytoplankton abundance compared to the control at week 18. Compared to individual insecticide applications, phytoplankton abundance exposed to mixtures was less resistant compared to exposure to the low concentration of diazinon, high concentration of endosulfan, and high concentration of malathion treatments.

#### **A.1.15 Periphyton**

The abundance of periphyton on the clay tiles was not affected by the pesticide treatments, but it did increase over time with no time-by-pesticide interaction (Table A4). However, we did notice that mesocosms that received the mixture and the high concentration of endosulfan contained large amounts of floating filamentous algae (J. Hua & R. Relyea, pers. obs.).

#### **A.1.16 Response of detritivores**

Since detritivore abundance was only sampled once, we assessed the response but not the recovery of these taxa to the pesticide treatments. The abundance of detritivores was affected by the pesticide treatment (Table A5). At the low concentration, detritivores were resistant to all insecticides. At the high concentration, detritivores were resistant to chlorpyrifos and diazinon but experienced reductions in abundance with malathion and endosulfan. The mixture of all four insecticides caused a large reduction in abundance exceeding the effects of chlorpyrifos,

diazinon, and malathion at the low concentration. The effect of mixtures was similar in effect to endosulfan and malathion at the high concentration but exceeded the effects of the high concentrations of diazinon and chlorpyrifos. To better understand these overall changes in the detritivore assemblage, we examined detritivores at the species level and assessed the response of amphipod and isopod abundance to these insecticides (Table A5).

#### **A.1.17 Amphipods**

The abundance of amphipods was affected by the pesticide treatment (Table A5C). At the low concentration, amphipods were resistant to diazinon, malathion, and endosulfan but chlorpyrifos completely eliminated the amphipods (Fig. 3). At the high concentration, the amphipods were severely reduced by malathion and completely eliminated by chlorpyrifos, diazinon, and endosulfan. The mixture of all four insecticides also eliminated the amphipods, similar to chlorpyrifos at the low concentration, but more than the individual effects of the low concentration of diazinon, endosulfan, and malathion. The reduction in amphipods by the mixture treatment was similar to the individual effects of each insecticide at the high concentration.

#### **A.1.18 Isopods**

The abundance of isopods was affected by the pesticide treatment (Table A5C; Fig. 3). At the low concentration, isopods were resistant to chlorpyrifos, diazinon, and malathion but endosulfan completely eliminated the isopods. Similarly, at the high concentration, isopods were resistant to chlorpyrifos, diazinon, and malathion but endosulfan completely eliminated the isopods. The

mixture of all four insecticides caused an elimination of isopods similar to diazinon and endosulfan at the low concentration but this reduction was greater than the individual effects of the low concentrations of chlorpyrifos and malathion. The reduction in isopods by the mixture treatment was similar to the individual effects for chlorpyrifos, endosulfan, and malathion but exceeded the effect of diazinon at the high concentration.

#### **A.1.19 Response of snail assemblage**

The abundance of snails was not affected by any pesticide treatment. All three snail species were resistant to both individual insecticides applications and mixtures (Table A6).

#### **A.1.20 Amphibian survival**

Given the 18-wk duration of this experiment and the terrestrial life history of amphibians, we could assess the response but not the recovery of amphibians. To better understand the overall changes in total amphibian survival, we start by examining the spring-breeding amphibians by assessing wood frogs, leopard frogs, American toads, and spring peeper survival in the insecticide treatments. The survival of wood frogs, leopard frogs, and American toads were affected by the pesticide treatment while spring peepers were never affected (Table A7). At the low concentration, all four species were resistant to chlorpyrifos, malathion, and endosulfan while diazinon caused an decrease in American toad survival (Fig. 4). At the high concentration, all four species were resistant to chlorpyrifos, diazinon, and malathion, However, endosulfan caused a sharp reduction in the survival of American toads and the complete elimination of wood frogs and leopard frogs. The insecticide mixture reduced the survival of leopard frogs and



American toads compared to the control. For leopard frogs, survival in the mixture was lower than the individual effects of chlorpyrifos, diazinon, and malathion at both the high and low concentration. For American toads, survival in the mixture was lower than the effects of malathion and endosulfan at the low concentration.

For the summer-breeding amphibians, we only assessed gray tree frogs survival to metamorphosis because the green frogs had to overwinter. The survival of gray treefrogs was affected by the pesticide treatment (Table A7). At the low concentrations, gray treefrogs were resistant to all insecticides but the high concentration of malathion caused a small reduction in survival (Fig. 4). The mixture of all four insecticides had no effect on gray treefrog survival compared to the control.

#### **A.1.21 Amphibian mass**

To understand the effect of insecticides on amphibian mass, we began by examining the masses of the spring-breeding amphibians. Univariate analyses indicated that the masses of leopard frogs and spring peepers were affected by the pesticide treatment while the masses of wood frogs and American toads were resistant (Table A8). At the low concentration, the masses of all four species were resistant to all insecticides (Fig. 5). At the high concentration, all four species were resistant to diazinon, malathion, and endosulfan, but chlorpyrifos caused an increase in spring peeper mass. The insecticide mixture caused an increase in leopard frog and spring peeper mass compared to the control. When exposed to the mixture, leopard frog mass was similar to the high concentration of chlorpyrifos, malathion and endosulfan. However, it exceeded the mass of leopard frogs exposed to the low concentration of chlorpyrifos, both the low and high concentrations of diazinon, the low concentration of malathion, and the low concentration of

endosulfan. When exposed to the mixture, spring peepers mass was similar to the low concentration of chlorpyrifos and diazinon and the high concentration of chlorpyrifos, endosulfan, and malathion. However, it exceeded the mass of peepers exposed to the low concentration of malathion and endosulfan and high concentration of diazinon.

For the summer-breeding amphibians, we measured the mass of gray treefrog metamorphs and green frog tadpoles. Univariate analyses indicated that the mass of both species was affected by the pesticide treatment (Table A8; Fig. 5). At the low concentration, both species were resistant to all four insecticides compared to the control. At the high concentration, both species were resistant to diazinon, malathion, and endosulfan, but chlorpyrifos caused an increase in gray treefrog mass. The insecticide mixture caused an increase in gray treefrog mass but not green frog mass compared to the control. The mass of gray treefrogs in the mixtures was similar to the low concentration of chlorpyrifos, diazinon, and endosulfan and the high concentration of chlorpyrifos, endosulfan, and malathion. However, it exceeded the mass of those individually exposed to the low concentration of malathion and the high concentration of diazinon. The mass of green frog tadpoles exposed to the mixture was significantly heavier than those exposed to the low concentration of diazinon.

#### **A.1.22 Time to metamorphosis**

For the spring breeding amphibians, leopard frogs and spring peepers were affected by pesticides while wood frogs and American toads were resistant. For the summer-breeding amphibians, gray treefrogs were affected by pesticides (Table A9; Fig. 6).

At the low concentration, leopard frogs metamorphosed earlier in chlorpyrifos and diazinon but they were resistant in malathion and endosulfan. At the high concentration, leopard

frogs metamorphosed earlier when exposed to malathion but were resistant to chlorpyrifos and diazinon. There was no effect of the mixture.

For peepers, there was no effect of insecticides applied individually on time to metamorphosis compared to the control. However, time to metamorphosis in the insecticide mixture caused peepers to metamorphose earlier than the low concentration of endosulfan and malathion and the high concentration of diazinon and endosulfan.

For gray treefrogs, time to metamorphosis was resistant to all insecticides at the low concentration compared to the control. At the high concentration, gray tree frogs exposed to chlorpyrifos and endosulfan completed metamorphosis faster while those exposed diazinon and malathion were resistant. The mixture caused earlier metamorphosis than the control, the low concentration of diazinon and malathion, and the high concentration of diazinon.

#### **A.1.23 Indirect effect of endosulfan on cladoceran abundance**

When exposed to the low concentration of endosulfan, we found that cladoceran abundances were significantly more abundant relative to cladoceran abundances in the control (Fig. 1; Table A4C). In contrast, the same endosulfan treatment dramatically depressed copepod abundances. Consideration of the ecological interactions between the zooplankton assemblages can provide insight to the mechanisms contributing to their polar responses to endosulfan. Cladocerans and copepods compete for phytoplankton resources (Sommers et al., 2008); as a consequence, the direct decimation of copepod by the low concentration of endosulfan indirectly facilitated cladocerans. Though the high concentrations of endosulfan also eliminated copepod abundances, cladocerans in the high endosulfan treatment were not significantly higher relative to the control. This suggests that the ability for cladoceran abundances to increase may be inhibited by the

higher concentration of endosulfan. Though not lethal, many studies have shown that insecticides can have negative sublethal consequences on population growth (Stark and Banks, 2003). Future studies considering sublethal impacts can provide important contributions to our understanding of insecticide risk.

#### **A.1.24 Sublethal effects on amphibian time to metamorphosis**

In addition to lethal effects, the insecticides also had sublethal effects on amphibian time to metamorphosis. The process of amphibian metamorphosis is environment- and species-dependent and amphibians can initiate adaptive metamorphic responses to escape unfavorable larval conditions (Newman, 1992; Skelly, 1996). In our study, leopard frogs exposed to either the low concentration of chlorpyrifos and diazinon or the high concentration of malathion and gray treefrogs exposed to high concentrations of chlorpyrifos and endosulfan metamorphosed faster relative to animals in the control (Fig A5, Table A9). Past studies have demonstrated a large disparity in the effects of insecticides on amphibian time to metamorphosis. For instance, (Distel and Boone, 2009) found that the insecticide carbaryl led to increase time to metamorphosis of American toads, (Relyea and Diecks, 2008) found no effect on wood frogs and an increase in leopard frog time to metamorphosis, and (Relyea, 2009) found no effect of carbaryl, malathion, chlorpyrifos, diazinon, and endosulfan on leopard frog time to metamorphosis. These studies all consider the effects of insecticides at different concentrations and timing of application. Future work is needed to determine how metamorphosing strategies of amphibians in response to insecticides can change depending on timing and frequency of insecticide application. To sum, the integration of ecological interactions with chemical toxicity is critical to understanding amphibian responses to insecticides.

### **A.1.25 Defining additive and non-additive effects**

To assess the consequences of mixtures, we used the same criteria described by Hayes et al., 2006. We first determined whether there was an overall significant effect of insecticide treatment using a series of ANOVA test (described in detail in the statistical analysis section of the main text; Section 2.7). For all significant ANOVA results, we used pairwise comparisons (specific tests detailed in Section 2.7) to compare the responses of taxa to insecticides applied individually versus applied as a mixture (Table A12). We began by comparing the effects of pesticide mixtures to the effects of the insecticides on each taxa applied individually at the additive concentration (low concentration treatment). Then, to confirm that these responses were not due to the higher total concentration of the mixture treatment, we also examined the response of the various taxa to individual applications of the substitutive concentration (high concentration treatments).

We defined synergistic effects as responses to the insecticide mixture that are significantly greater than the responses caused by insecticides applied individually at the additive concentration. Then, to confirm that these responses were not due to the higher total concentration of the mixture treatment, we also examined the response of the various taxa to individual applications of the substitutive concentration (high concentration treatments). In contrast, antagonistic effects occur when the response to insecticide mixture is less than the additive response of insecticides applied individually (Darling and Cote 2008).

#### **A.1.26 Detailed results of insecticides applied as a mixture**

We found 17 cases where insecticides applied in a mixture had more severe consequences compared to individually applied insecticides (Figures 1-6; A2-A4). In particular, the mixture more severely reduced cladoceran and copepod abundance compared to all four insecticides applied individually at the additive concentration (Table A12). Mixtures also significantly reduced amphipod abundances compared to the additive concentrations of diazinon, malathion, and endosulfan and significantly reduced isopod abundances compared to the additive concentration of chlorpyrifos and malathion. Finally for amphibians, mixtures reduced leopard frog survival significantly more than chlorpyrifos and diazinon applied individually and mixtures reduced American toad survival more than endosulfan and malathion applied individually. In all these cases, the substitutive concentration (high concentration treatments) of individual insecticides never had a significantly larger impact than the mixture treatment at equivalent concentrations. Thus, we can confirm that the highly severe effects of mixtures were not due to the higher total concentration of the mixture treatment (Table A12).

## APPENDIX B

### ADDITIONAL INFORMATION (CHAPTER 2 TABLES AND FIGURES)

**Table 6-1. Properties of the four pesticides used in the experiment, including half-life in water, maximum aquatic concentrations that have been detected in nature, and LC-50 values for zooplankton and amphibians.**

<b>Insecticide</b>	<b>Chemical class and mode of action</b>	<b>Half life (wks)</b>	<b>Average and maximum concentrations in water bodies (ug/L)</b>	<b>LC-50 for zooplankton (ug/L)</b>	<b>LC-50 for amphibians (ppm)</b>
Chlorpyrifos	Organophosphate- AChE inhibitor	2.2 to 4.3	6.7; 40.6	0.1- 1	0.09- 3
Diazinon	Organophosphate AChE inhibitor	6 to 24	162; 468	0.25-20	0.44-7.5
Malathion	Organophosphate AChE inhibitor	0.2 to 21	44.2; 583	1-5	0.2- 5.2
Endosulfan	Organochlorine GABA inhibitor	1.5 to 26	0.17; 23.8	24-250	0.009-0.112

**Table 6-2. Anuran egg collection data and initial masses of the tadpoles used in the experiment.**

<b>Breeding season</b>	<b>Species</b>	<b>Date collected</b>	<b>Source pond</b> (41° N, 80° W)	<b># of egg masses collected</b>	<b>Initial mass</b> <b>± 1 SE (mg)</b>
Spring	Wood frogs	20-28 March	Mallard	14	91.6 ± 5.6
Spring	Leopard frogs	2-4 April	Mallard	11	67.3 ± 3.5
Spring	American toads	22-26 April	Oberdick	16	20.8 ± 1.1
Spring	Spring peepers	27-28 April	Farm Lab	20	19.4 ± 1.7
Summer	Gray treefrogs	2-4 May	Farm Lab	14	25.0 ± 1.4
Summer	Green frogs	28 May- 6 June	Oberdick, Love, Geneva	15	11.3 ± 1.13



**Table 6-3. Results of a repeated-measures MANOVA on temperature, pH, dissolved oxygen, and light attenuation measured at four time points.**

A. Multivariate tests (Wilks' lambda)	df	F-value	P-value
Pesticides	36, 103	3.6	<b>&lt;0.001</b>
Time	12, 19	0.00	1.00
Pesticides * Time	108, 151	1.4	<b>0.019</b>

B. Univariate tests	Pesticides (df = 9, 30)	Time (df = 9, 30)	Pesticides * Time (df = 27, 90)
Temperature	0.231	1.0	0.11
pH	<b>&lt;0.001</b>	1.0	<b>0.015</b>
Dissolved oxygen	<b>&lt;0.001</b>	1.0	0.25
Light attenuation	<b>0.001</b>	1.0	<b>0.003</b>

C. Univariate tests within each sample time	pH (df = 9, 30)	Dissolved oxygen (df = 9, 30)	Light attenuation (df = 9, 30)
Week 2	<b>0.003</b>	<b>0.002</b>	<b>0.004</b>
Week 4	<b>&lt;0.001</b>	<b>0.03</b>	<b>0.011</b>
Week 9	<b>&lt;0.001</b>	<b>0.001</b>	<b>0.001</b>
Week 18	0.27	0.26	0.13

**Table 6-4. Results of a repeated-measures MANOVA on the abundance of cladocerans, copepods, phytoplankton, and periphyton measured at four time points.**

A. Multivariate tests (Wilks' lambda)	df	<i>F</i> -value	<i>P</i> -value
Pesticides	36, 103	5.8	<b>&lt; 0.001</b>
Time	12, 19	2.9	<b>0.03</b>
Pesticides * Time	2, 108	2.2	<b>&lt; 0.001</b>

B. Univariate tests	Pesticides (df = 9, 30)	Time (df = 3, 90)	Pesticides * Time (df = 27, 90)
Cladocerans	<b>&lt;0.001</b>	0.99	<b>&lt;0.001</b>
Copepods	<b>&lt;0.001</b>	1.00	<b>&lt;0.001</b>
Phytoplankton	<b>0.007</b>	1.00	<b>&lt;0.001</b>
Periphyton	0.91	<b>&lt;0.001</b>	0.34

C. Univariate tests within each sample time	Cladocerans (df = 9, 30)	Copepods (df = 9, 30)	Phytoplankton (df = 9, 30)
Week 2	<b>&lt;0.001</b>	<b>0.001</b>	0.54
Week 4	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Week 9	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Week 18	<b>0.003</b>	<b>&lt;0.001</b>	<b>0.01</b>

**Table 6-5. Results of (a) ANOVA on total detritivore abundance, (b) MANOVA on amphipod and isopod abundance, and (c) ANOVA on amphipod and isopod the abundances at week 13.**

<b>A. Univariate test (Total detritivore abundance)</b>			
	df	<i>F</i> -value	<i>P</i> -value
Pesticide	9, 30	5.09	<b>&lt; 0.001</b>
<b>B. Multivariate test (Wilks' lambda)</b>			
	df	<i>F</i> -value	<i>P</i> -value
Pesticide	18, 58	7.0	<b>&lt; 0.001</b>
<b>C. Univariate tests</b>			
	Amphipod abundance (df = 9, 30)	Isopod abundance (df = 9, 30)	
Pesticide	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	

**Table 6-6. Results of a repeated-measures MANOVA on the abundance of three snail species measured at two time points.**

A. Multivariate test (Wilks' lambda)	df	<i>F</i> -value	<i>P</i> -value
Pesticide	27, 82	2.6	0.09
Time	3, 28	0.0	1.00
Pesticide * Time	27, 82	1.33	0.16

**Table 6-7. Results of (a) MANOVA on survival of each species and (b) ANOVA on survival of each anuran species.**

A. Multivariate test (Wilks' lambda)		df	F-value	P-value	
Pesticide		45, 119	3.3	< <b>0.001</b>	
B. Univariate tests	Wood frogs (df = 9, 30)	Leopard frogs (df = 9, 30)	American toads (df = 9, 30)	Spring peepers (df = 9, 30)	Gray treefrogs (df = 9, 30)
Pesticide	<b>0.04</b>	<b>0.001</b>	<b>&lt;0.001</b>	0.61	<b>0.03</b>

**Table 6-8. Results of (a) MANOVA on mass of each anuran species at metamorphosis and (b) ANOVA on mass of each anuran species.**

A. Multivariate test (Wilks' lambda)	df	<i>F</i> -value	<i>P</i> -value
Pesticide	40, 50	2.4	<b>0.002</b>

B. Univariate tests	Wood frogs (df = 9, 30)	Leopard frogs (df = 9, 30)	American toads (df = 9, 30)	Spring peepers (df = 9, 30)	Gray treefrogs (df = 9, 30)	Green frogs (df = 9, 30)
Pesticide	0.18	<b>0.004</b>	0.25	<b>0.002</b>	<b>0.04</b>	<b>0.001</b>

**Table 6-9. Results of a MANOVA on time to metamorphosis for the five species of amphibians that metamorphosed.**

A. Multivariate test (Wilks' lambda)		df	F-value	P-value	
Pesticide		45, 119	2.9	< <b>0.001</b>	

B. Univariate tests	Wood frogs (df = 9, 30)	Leopard frogs (df = 9, 30)	American toads (df = 9, 30)	Spring peepers (df = 9, 30)	Gray treefrogs (df = 9, 30)
Pesticide	0.14	< <b>0.001</b>	0.36	< <b>0.001</b>	< <b>0.001</b>

**Table 6-10. Results of a repeated-measures MANOVA on the abundance of (a) Biotic variables- cladoceran, copepods, phytoplankton and periphyton and (b) Abiotic variables- temperature, pH, dissolved oxygen, and light attenuation exposed to H2O or Etoh control.**

A. Biotic variables	df	<i>F</i> -value	<i>P</i> -value
Treatment	4, 3	1.7	0.34
Time	12, 40	13.4	<0.001
Pesticide * Time	12, 40	1.1	0.39
B. Abiotic variables			
Treatment	4, 3	2.0	0.30
Time	12, 40	14.2	<0.001
Pesticide * Time	12, 40	1.3	0.25



**Table 6-11. Average and standard deviation of (a) Biotic variables and (b) Abiotic variables- exposed to H<sub>2</sub>O or ETOH controls.**

A. Biotic variables	H <sub>2</sub> O control	ETOH control <sup>1</sup>
Cladocerans	1.5 ± 0.23	1.4 ± 0.42
Copepods	0.76 ± 0.43	0.77 ± 0.43
Phytoplankton	0.99 ± 0.41	1.0 ± 0.34
Periphyton	1.9 ± 0.41	1.8 ± 0.31
B. Abiotic variables		
Temperature	20.9 ± 0.62	20.8 ± 0.69
pH	8.1 ± 0.15	8.1 ± 0.21
Dissolved oxygen	7.8 ± 0.85	7.7 ± 0.82
Light attenuation	0.007± 0.002	0.009 ± 0.003

**Table 6-12. Significant pairwise comparisons (P-values) between mixtures and insecticides applied individually.**

Taxa	Week	Chlorpyrifos		Diazinon		Endosulfan		Malathion	
		Low	High	Low	High	Low	High	Low	High
Cladocerans	2	<b>0.001</b>	<b>0.001</b>	<b>0.04</b>	0.07	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.001</b>	0.479
	4	0.11	0.85	1	0.6	<b>&lt;0.001</b>	<b>0.001</b>	<b>&lt;0.001</b>	0.373
	9	<b>0.006</b>	<b>0.025</b>	<b>0.006</b>	<b>0.025</b>	<b>&lt;0.001</b>	0.32	<b>0.015</b>	<b>&lt;0.001</b>
	18	<b>0.022</b>	1	<b>&lt;0.001</b>	0.3	<b>0.003</b>	<b>0.02</b>	<b>0.02</b>	0.123
Copepods	2	0.84	0.84	0.556	<b>0.002</b>	0.4	0.64	<b>0.004</b>	0.28
	4	0.52	1	<b>&lt;0.001</b>	0.076	0.124	0.086	0.208	<b>&lt;0.001</b>
	9	0.15	1	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.009</b>	0.82	<b>0.06</b>	<b>0.022</b>
	18	<b>0.014</b>	<b>0.014</b>	0.52	0.07	0.376	<b>0.014</b>	0.78	0.73
Amphipods	13	1	1	<b>0.005</b>	1	<b>0.015</b>	1	<b>0.001</b>	0.98
Isopods	13	1	1	<b>0.005</b>	1	<b>0.015</b>	1	<b>0.001</b>	0.98
Wood frogs	18	0.8	1	0.84	0.86	0.34	0.57	0.29	0.88
American toads	18	0.95	0.55	1	0.24	<b>0.001</b>	1	<b>0.002</b>	0.91
Spring peepers	18	1	0.87	0.99	1	0.38	1	1	0.69
Leopard frogs	18	<b>0.039</b>	0.089	<b>0.01</b>	<b>0.006</b>	0.55	1	0.55	<b>0.014</b>
Gray treefrog	18	0.93	0.97	0.3	0.58	0.97	0.44	<b>0.017</b>	<b>0.018</b>

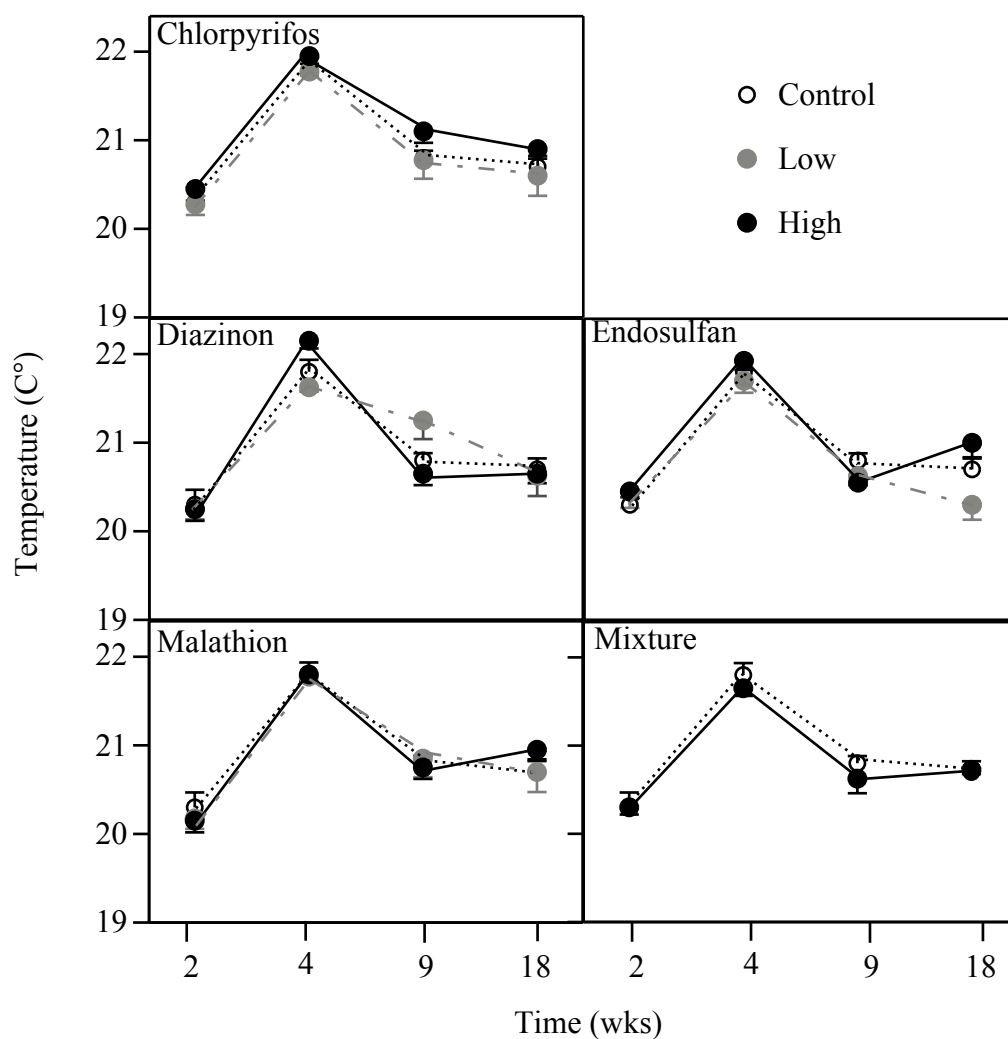
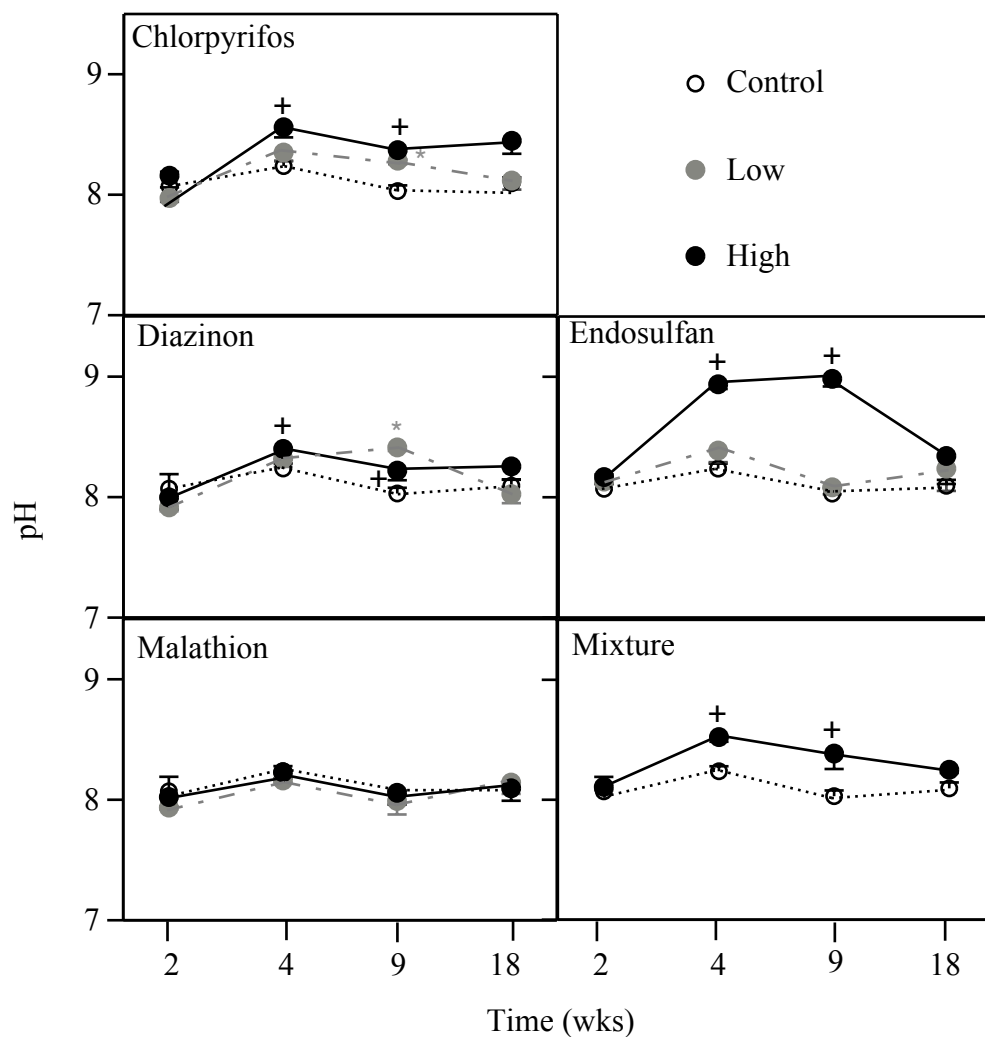


Figure 6-1. Effects of four insecticides, applied separately and in a mixture, on temperature at week 2, 4, 9, and 18. Data are means  $\pm$  SE. Symbols \* and + indicate significance at the  $p < 0.05$  for 10 ug/L and 40 ug/L, respectively, compared to the control.



**Figure 6-2. Effects of four insecticides, applied separately and in a mixture, on pH at week 2, 4, 9, and 18. Data are means  $\pm$  SE. Symbols \* and + indicate significance at the  $p < 0.05$  for 10 ug/L and 40 ug/L, respectively, compared to the control.**

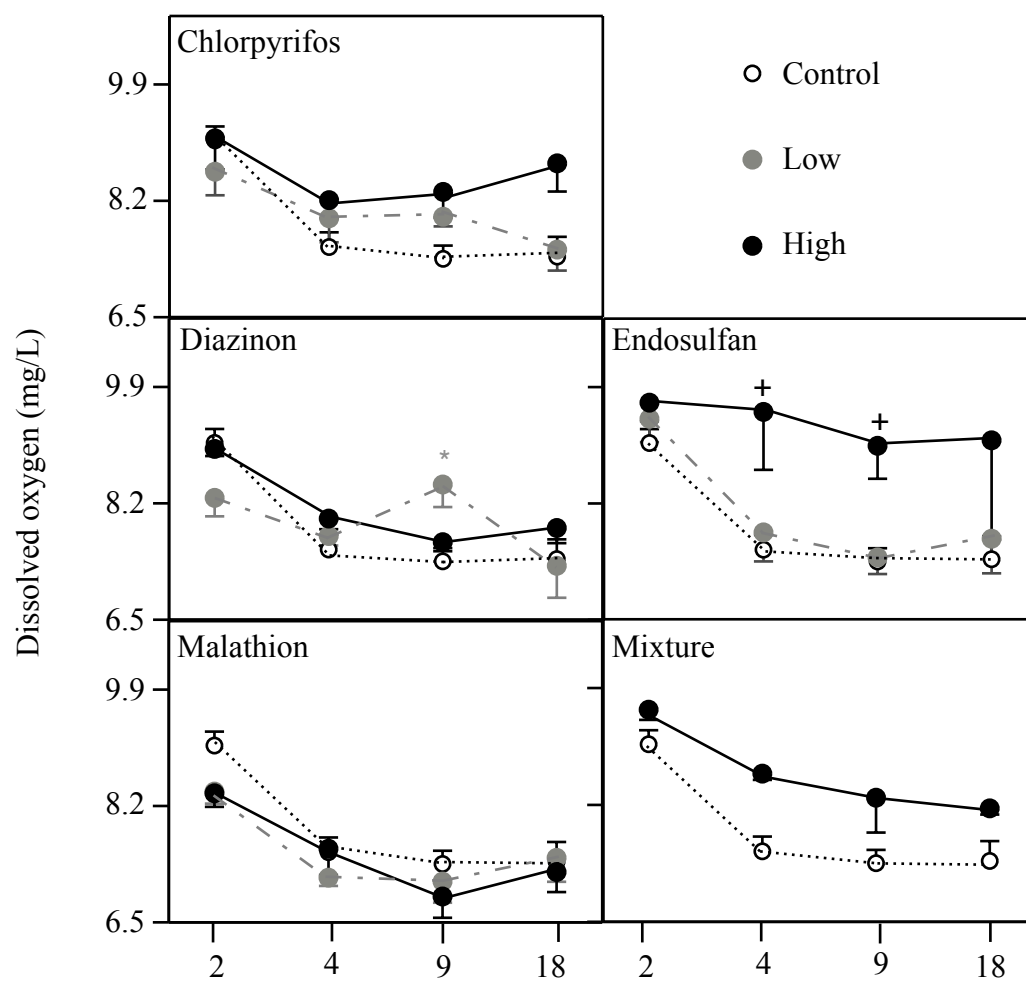


Figure 6-3. Effects of four insecticides, applied separately and in a mixture, on dissolved oxygen at week 2, 4, 9, and 18. Data are means  $\pm$  SE. Symbols \* and + indicate significance at the  $p < 0.05$  for 10 ug/L and 40 ug/L, respectively, compared to the control.

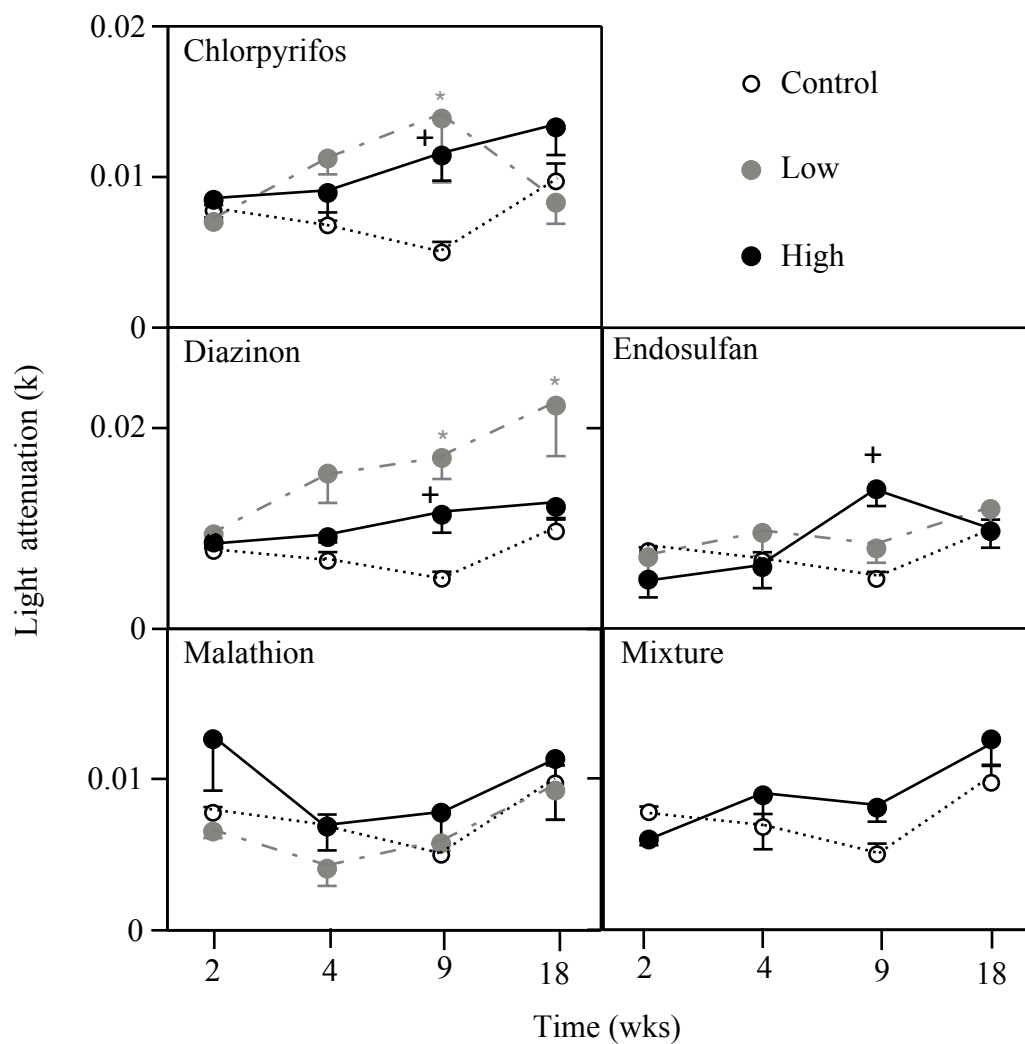
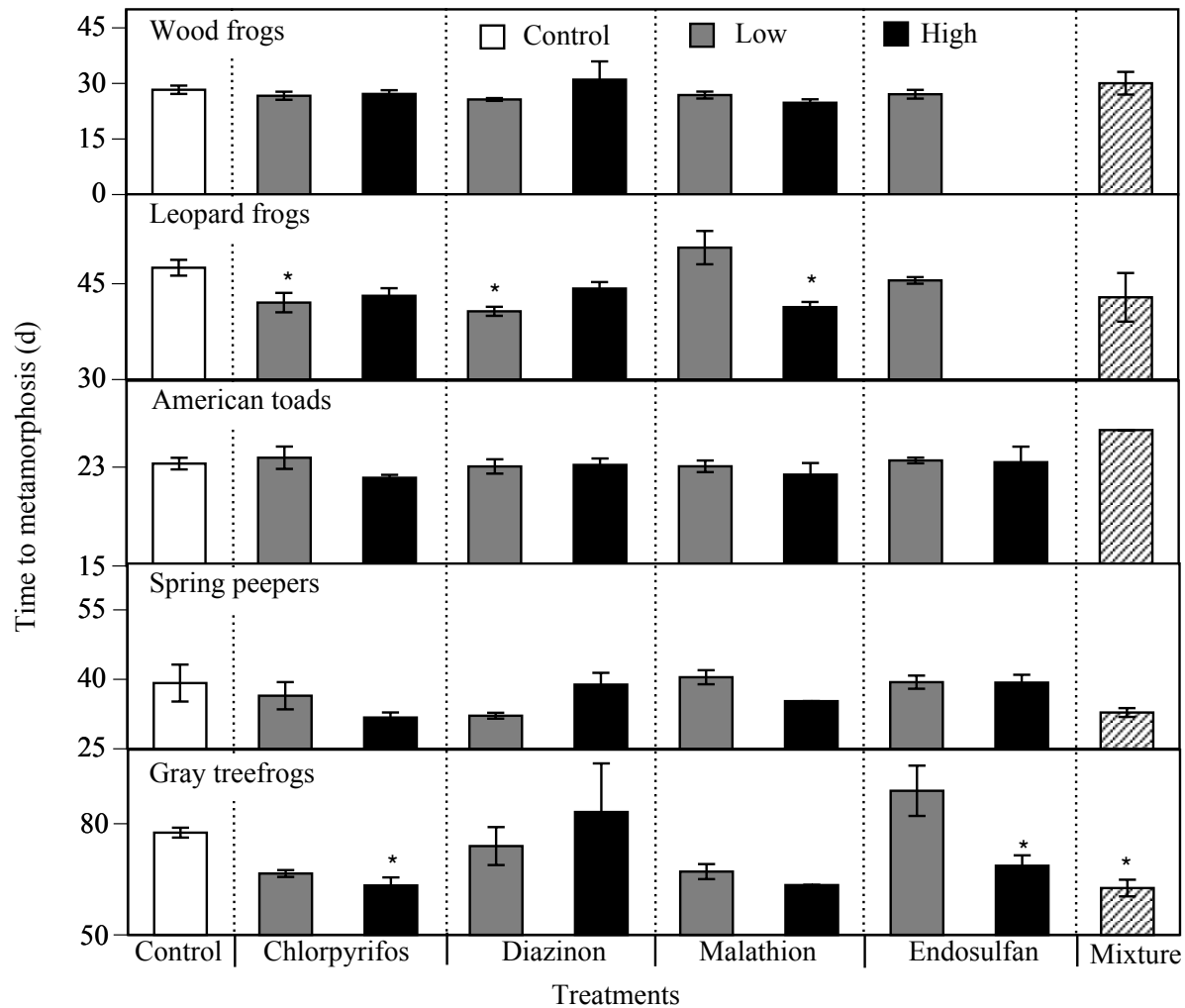


Figure 6-4. Effects of four insecticides, applied separately and in a mixture, on light attenuation (k) at weeks 2, 4, 9, and 18. Data are means  $\pm$  SE. Symbols \* and + indicate significance at the  $p < 0.05$  for 10 ug/L and 40 ug/L, respectively, compared to the control.



**Figure 6-5. Effects of four insecticides, applied separately and in a mixture, on time to metamorphosis of wood frog, American toad, spring peeper, leopard frog, and gray tree frog time to metamorphosis . Data are mean  $\pm$  SE. Asterisks indicate treatments that differ from the control  $p < 0.05$**

## APPENDIX C

### ADDITIONAL FIGURES AND TABLES (CHAPTER 3)

**Table 6-13 Properties of the three pesticides used in the experiment, including half-life in water, maximum aquatic concentrations that have been detected in nature, and LC-50 values for amphibians.**

<b>Insecticide</b>	<b>Half life (wks) <sup>1</sup></b>	<b>Maximum detection (ppb) in nature</b>	<b>LC-50 for amphibians (ppm) <sup>2</sup></b>
Carbaryl	1.4 - 28	2500 <sup>3</sup>	2.1- 26.1
Chlorpyrifos	2.2 to 4.3	5.8 <sup>4</sup>	0.6- 3
Malathion	0.2 to 21	583 <sup>5</sup>	1.3- 5.9



**Table 6-14 Properties of the three pesticides used in the experiment, including half-life in water, maximum aquatic concentrations that have been detected in nature, and LC-50 values for amphibians. A) Insecticides were dissolved in ETOH solvent. ETOH concentrations were below the ASTM solvent standard for aquatic test species. We chose not to incorporate a solvent control since past studies have demonstrated that solvent concentrations higher than we used did not effect tadpoles. B) Samples were assessed 3 months after the experiment, thus the samples likely experienced some degree of breakdown prior to testing.**

<b>Insecticide</b>	<b>Stock solution concentration (ppm)</b>	<b>Volume (uL) of Stock solution added to 6.5 L<sup>a</sup></b>	<b>Nominal concentration (ppm)</b>	<b>Actual concentration (ppm)<sup>b</sup></b>
Carbaryl	5000	7.8	6	1.9
Chlorpyrifos	2024	5.8	1.75	0.3
Malathion	5000	13	10	0.4

**Table 6-15. Anuran egg collection data and initial masses of the tadpoles used in the experiment.**

<b>Pond</b>	<b>Date collected</b>	<b>County</b>	<b>Source pond</b>	<b>Relative distance to *</b>
Blackjack*	4 April	Crawford	(41.66° N, 80.51° W)	-
Mallard	4 April	Crawford	(41.69° N, 80.50° W)	4.8 km
Staub	7 April	Crawford	(41.59° N, 80.43° W)	21.2 km
Relyea	6 April	Crawford	(41.62° N, 80.45° W)	22.9 km
Trailer Park	7 April	Crawford	(41.57° N, 80.45° W)	23.3 km
Graveyard	5 April	Crawford	(41.68° N, 80.04° W)	47.5 km
Turkey Track	4 April	Crawford	(41.63° N, 79.91° W)	72.3 km
Square*	7 April	Erie	(41.84° N, 80.24° W)	-
Hopscotch	4 April	Erie	(41.87° N, 80.47° W)	22.7 km
Boro	11 April	Erie	(41.92° N, 80.03° W)	24.7 km
Reed	11 April	Erie	(41.98° N, 79.96° W)	37.9 km
Bowl	11 April	Erie	(41.92° N, 79.80° W)	45 km
Skinny	11 April	Erie	(41.99° N, 79.78° W)	55.8 km
Log*	11 April	Warren	(41.97° N, 79.59° W)	-
Road	11 April	Warren	(41.88° N, 79.60° W)	12.9 km

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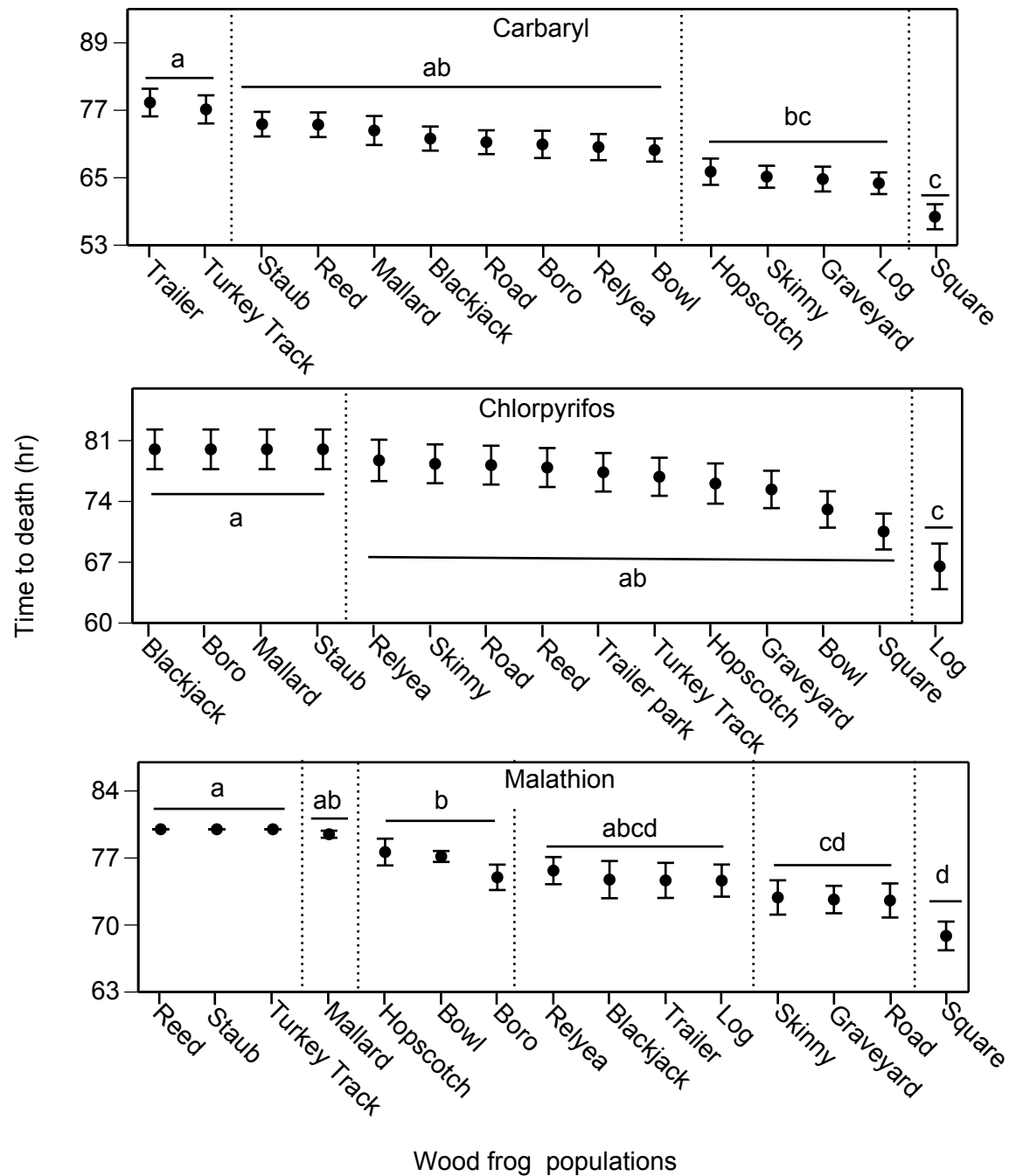


Figure 6-6. Effects of carbaryl, chlorpyrifos, and malathion on average tadpole time to death. Data are means  $\pm$  SE. Values sharing similar letters represent statistically similar values ( $p > 0.05$ ).

## APPENDIX D

### ADDITIONAL INFORMATION (CHAPTER 4)

#### D.1.1 Results from the analysis of variance

*Constitutive tolerance of tadpoles from the four populations:* To determine the constitutive tolerance of the four populations, we conducted a univariate analysis on the TTD of tadpoles exposed to 0 ppb in Phase 1 and the lethal concentration of carbaryl in Phase 2. Since these tadpoles were not subjected to insecticides in Phase 1, these TTD data provide an estimate of constitutive tadpole tolerances from each of the populations. Since tadpoles from the embryo and hatchling experiment faced different rearing conditions, we conducted separate ANOVAs for each experiment.

For the embryo experiment, we found that tadpole TTD did not differ between populations close to agriculture and populations far from agriculture ( $F_{1, 18} = 0.136$ ;  $p = 0.71$ ). However, tadpoles from populations close to agriculture tended to survive longer than those far from agriculture (Table A2). For the hatchling experiment, we found that tadpole TTD was higher for populations close to agriculture compared to populations far from agriculture ( $F_{1, 18} = 4.5$ ;  $p = 0.049$ ).

Based on these analyses, we conclude that tadpoles from populations close to agriculture were more tolerant to carbaryl than populations far from agriculture but this higher tolerance was only observed in the hatchling experiment. Tadpoles in both the embryo and hatchling-exposure experiment consisted of individuals from the same mixture of egg masses but tadpoles from these experiments varied in their rearing condition. Tadpoles from the embryo-exposure experiment were held indoors at a constant temperature of 20°C throughout the duration of the experiment while tadpoles from the hatchling were temporarily held outdoors for 7 d (Fig. 1) where outdoor temperatures fluctuated between 11 to 24°C. Thus, it is possible that the variation in rearing condition could have accounted for the variation in standing tadpole tolerance in the embryo and hatchling exposure experiments. Overall, though not always significant, populations close to agriculture had higher constitutive tolerance compared to populations far from (Table A2).

**Survival of tadpoles during the TTD after being exposed to carbaryl as embryos-** In the analysis of tadpole survival during the TTD assay, we found a significant effect of population, concentration, and a population-by-concentration interaction (Table A3). Because of the interaction, we ran separate ANOVAs for each population. The univariate ANOVA demonstrated that embryonic exposure to carbaryl only affected the survival of tadpoles from the more susceptible populations that were also farther from agriculture (i.e. Hopscotch and Square Pond; Table A2). Tadpoles from Hopscotch pond experienced lower mortality if they had been previously exposed to 0.5 or 1 ppm of carbaryl as embryos ( $F_{3,16} = 3.5$ ;  $P = 0.04$ ). In contrast, tadpoles from Square pond experienced higher mortality if they had been previously exposed to 1 ppm of carbaryl as embryos (Fig. A1;  $F_{3,16} = 13.9$ ;  $P < 0.001$ ).

### **Survival of tadpoles during the TTD after being exposed to carbaryl as hatchlings-**

In the analysis of tadpole survival during the TTD assay, we found a significant effect of population, but no effect of concentration or a population-by-concentration interaction (Table A3). When we conducted a Tukey's pairwise test, we found that Square Pond was significantly different from Hopscotch ( $p = 0.002$ ), Staub ( $p = 0.001$ ), and Trailer park ( $p = 0.002$ ) ponds.

## APPENDIX E

### ADDITIONAL TABLES AND FIGURES (CHAPTER 4)

Table 6-16. Background information on each of the four populations.

Population	Percent agriculture (200 m radius)	Distance to closest agriculture	Tadpole tolerance <sup>1</sup>	GPS Location
Staub	18.6 %	97 m	74.4 ± 2.6	41.59° N, 80.43° W
Trailer Park	9.5 %	62 m	77.2 ± 2.5	41.57° N, 80.45° W
Hopscotch	0 %	879 m	64.0 ± 1.9	41.87° N, 80.47° W
Square	0 %	1264 m	58.8 ± 2.2	41.84° N, 80.24° W

1. Mean time to death (hrs) ± SE of tadpoles following exposure to 6 ppm of carbaryl are based on the experiments of Hua et al. (2013).



**Table 6-17. Tadpole tolerance to carbaryl (time to death) from populations close and far from agriculture in the absence of a previous sublethal exposure to carbaryl.**

<b>Proximity to agriculture</b>	<b>Embryo-experiment</b>	<b>Hatchling-experiment<sup>†</sup></b>
Far	138.84 ± 10.9 hrs	42.5 ± 7.3 hrs
Close	146.2 ± 4.5 hrs	62.4 ± 3.3 hrs

**Table 6-18 Survival of tadpoles exposed to 0 ppm of carbaryl in the TTD assay for the Embryo-exposure and hatchling-exposure experiments.**

Survival in TTD assay of control treatments			Phase 2			
			Embryo-exposure		Hatchling-exposure	
Proximity to agriculture	Population	Phase 1 treatment	Control		Control	
			survival (%)	SE	survival (%)	SE
Far	Hopscotch	0	98	2	84	11.2
		0.1	96	4	90	7.7
		0.5	98	2	90	6.3
		1	98	2	92	5.8
	Square	0	100	0	96	2.4
		0.1	100	0	92	8
		0.5	96	4	96	2.4
		1	100	0	98	2
Close	Staub	0	100	0	100	0
		0.1	94	4	98	2
		0.5	100	0	96	2.4
		1	98	2	86	14
	Trailer park	0	100	0	98	2
		0.1	100	0	100	0
		0.5	98	2	100	0
		1	98	2	100	0

**Table 6-19. Test results from ANOVAs on survival of tadpoles exposed to the lethal concentration during the TTD assay for A) the embryo-exposure experiment, B) the hatchling exposure experiment.**

		df	F	<i>P</i> -value
A. Embryo-exposure experiment	Population	3, 64	7.6	<b>&lt;0.001</b>
	Concentration	3, 64	3.4	<b>0.022</b>
	Pop'n x Conc	9, 64	2.2	<b>0.036</b>
B. Hatchling-exposure experiment	Population	3, 64	7.96	<b>&lt;0.001</b>
	Concentration	3, 64	0.45	0.72
	Pop'n x Conc	9, 64	0.59	0.79

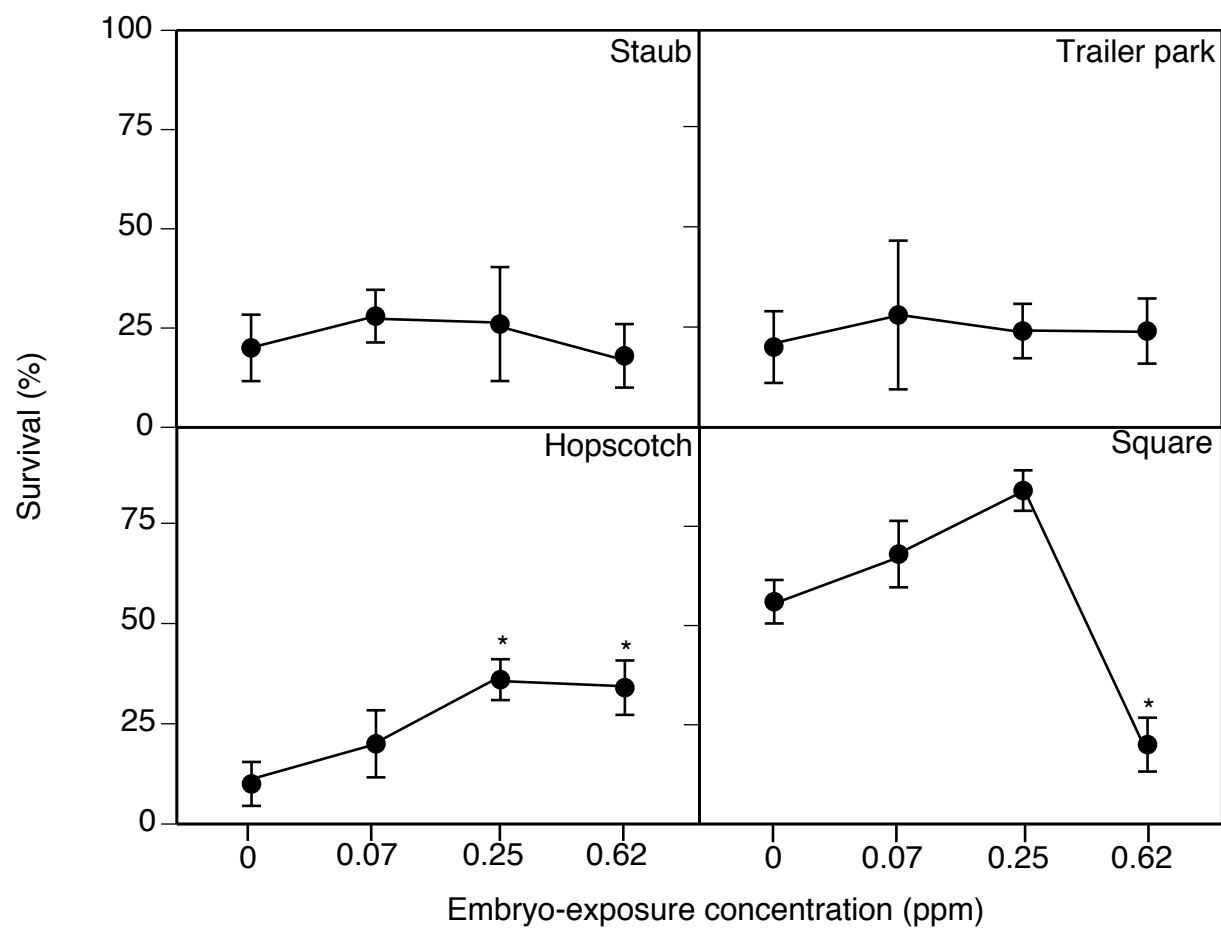


Figure 6-7. Survival of tadpoles exposed to four sublethal concentrations of carbaryl as embryos (0, 0.07, 0.25, and 0.62 ppm) followed by an exposure to a lethal concentration as tadpoles during the TTD assay.

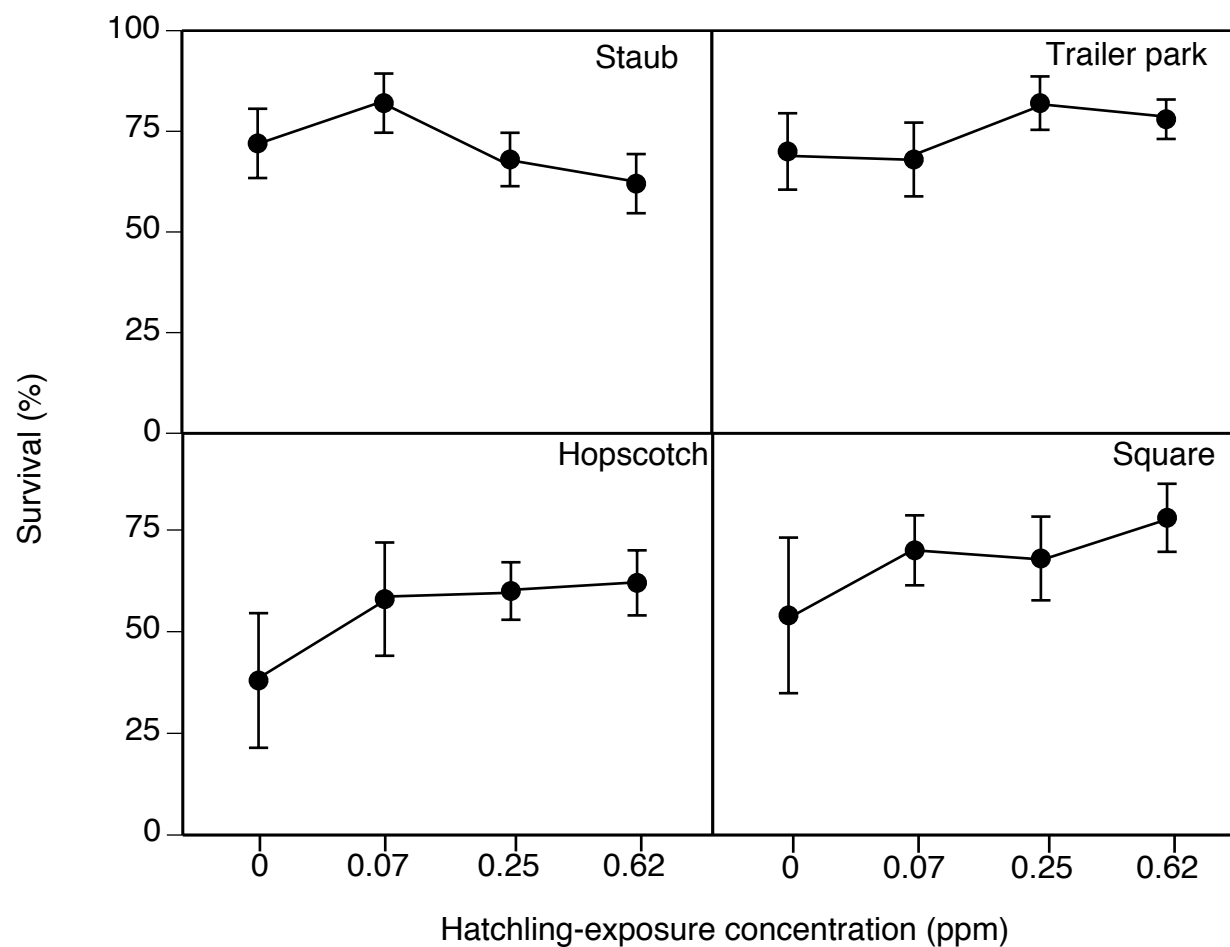


Figure 6-8. Survival of tadpoles exposed to four sublethal concentrations of carbaryl as hatchlings (0, 0.07, 0.25, and 0.62 ppm) followed by an exposure to a lethal concentration as tadpoles during the TTD assay.

## **APPENDIX F**

### **ADDITIONAL INFORMATION (CHAPTER 5)**

#### **F.1.1 Insecticide applications**

For Phase 1 (induction of tolerance), we created a working solution by dissolving a commercial grade solution of carbaryl (22.5% Sevin©) in filtered water (pH = 7). To achieve 0.5 and 1 mg/L of carbaryl, we added 7.5 and 15 ul of commercial grade carbaryl to 3.5 L of filtered water, respectively. We added 450 ml of the carbaryl solution to each of the 500-ml experimental units.

For Phase 2, we first dissolved technical-grade insecticides (malathion, chlorpyrifos, and permethrin) into an ETOH vehicle (Table S1) to create stock solutions. We did not include an ETOH vehicle control in this study since past studies have demonstrated that solvent concentrations higher than we used do not affect tadpole mortality. To prepare the working solutions of each insecticide, we added the concentrated stock solutions of malathion, chlorpyrifos, and permethrin or the formulated product of carbaryl and cypermethrin to filtered water (Table S1). We then added 70 ml of these working solutions to each of the Petri dishes. Finally, we used filtered water to create the control solutions.

### **F.1.2 Pesticide testing**

To determine the actual concentrations of the pesticides used in this study, we collected 500-mL samples of each working solution after animals were added in Phase 1 and after tadpoles were added into Petri dishes at Phase 2. Samples were stored in amber jars and kept at 4°C in accordance to established analytical methods (OECD 2007). Within 24 hrs of dosing, samples were shipped to an independent laboratory (Center for Environmental Sciences and Engineering, Storrs, CT, USA). For all pesticides except permethrin, we used identical methodologies for both populations, thus we only sent samples from Hopscotch Pond to be tested. Since we used different permethrin concentrations for each population, we sent a sample from each population to be tested. Actual concentrations for Phase 1 and 2 can be found in Table S2.

Despite storing samples in accordance to established analytical methods (OECD 2007), the samples were not tested at the analytical lab until 8 wks after their arrival. As a result, likely due to sample degradation, the actual concentrations of the Phase 2 lethal doses were lower than nominal concentrations (Sherma and Beroza 2005). Given the discrepancy between the actual and nominal concentrations, we conducted an additional experiment to confirm that the differences in actual and nominal concentrations were due to sample degradation and not a flaw in our experimental protocol. Actual concentrations for samples from the additional experiment can be found in Table S2. The samples from the additional experiment were held for 14 d prior to testing so they also experienced degradation. However, the difference between the actual and nominal concentrations for all insecticides was reduced except for malathion and chlorpyrifos. For the malathion sample, isomers of malathion degradates were detected in the sample jar indicating that the reported actual concentration was lower than nominal concentrations due to degradation within the sample jar during storage. For chlorpyrifos, the original and additional

chemical analysis detected the same actual concentration of 0.3 ppb. These consistent results suggest that our protocol was precise but there was likely degradation in our technical grade chlorpyrifos stock (pers.comm. Dr. Chris Perkins Laboratory Director at the UCONN Center for Environmental Sciences and Engineering). Similarly, for cypermethrin the consistently low actual concentration suggests degradation of our technical grade cypermethrin stock.



## APPENDIX G

### ADDITIONAL TABLES (CHAPTER 5)

**Table 6-20. Working solution preparation. Technical grade (T.G.) chemicals were first emulsified in ethanol (ETOH) prior to application in water, whereas formulated products (F.P.) were applied directly to water. All technical grade insecticides were purchased from Chem Services (West Chester, PA).**

<b>Insecticide</b>	<b>Formulation, Purity</b>	<b>Stock solution concentration</b>	<b>TG insecticide added to 5 ml ETOH</b>	<b>Working solution concentration</b>	<b>Stock solution added to 1.5 L water</b>
Carbaryl	FP, 22.5%	22.5% a.i.	--	15 mg/L	1.13 ml
Chlorpyrifos	TG, > 98%	0.02 g/ml	0.1 g	5 mg/L	375 uL
Malathion	TG, > 95%	0.02 g/ml	100 ul	15 mg/L	0.9 ml
Cypermethrin	FP, 26%	26% a.i.	--	30 µg/L	0.8 uL
Permethrin	TG, > 98%	0.0067 g/ml	0.1 g	100 µg/L	22.5 uL

**Table 6-21. Chemical analysis results using both gas and liquid chromatography-tandem mass spectrometry. Formulations used in Phase 1 and 2 were either formulated products (FP) or technical grade (TG) chemicals. Technical grade chemicals were first emulsified with 95% ethanol prior to application.**

Insecticide treatment	Formulation	Nominal concentration	Actual concentration	Actual concentration
		(mg/L)	(mg/L)	(mg/L)
			1 <sup>st</sup> analysis	2 <sup>nd</sup> analysis
<i>Phase 1</i>				
Control	-	0.0	0.0	Did not retest
Carbaryl	FP	0.5	0.53	Did not retest
Carbaryl	FP	1.0	1.30	Did not retest
<i>Phase 2</i>				
Control	-	0.0	0.0	0.0
Carbaryl	FP	15	5.0	13.0
Chlorpyrifos	TG	5	0.3	0.3
Malathion	TG	15	5.7	1.9
Cypermethrin	FP	0.03	0.002	0.008
Permethrin (Hopscotch)	TG	0.1	0.014	0.06
Permethrin (Square)	TG	0.5	0.026	0.2

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